IDENTIFICATION OF AMINO ACID-STARVATION INDUCED mRNAs IN Fao RAT HEPATOMA CELLS

by

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To Betsy and Laura.

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ABBREVIATIONS

bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
IPTG	isopropylthio-B-D-galactopyranoside
kb	kilobases
MOPS	3-(N-morpholino) propanesulfonic acid
PAGE	polyacrylamide gel electrophoresis
RNase	ribonuclease
SDS	sodium dodecyl sulfate
SDS-PAGE	polyacrylamide gel electrophoresis with sodium dodecyl sulfate
SSC	standard sodium citrate buffer
SSPE	standard sodium phosphate/EDTA buffer
TBE	Tris/boric acid/EDTA buffer
TE	Tris/EDTA buffer
X-gal	5-chloro-4-bromo-3-indolyl B-D-galactopyranoside

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Chairman: Dr. Michael S. Kilberg
Major Department: Biochemistry and Molecular Biology

Total and subtracted lambda gt11 cDNA libraries were derived from rat Fao hepatoma cells which had been starved for amino acids for 3 hours. These libraries were screened using the method of differential hybridization or "plus-minus" screening to identify cDNA clones corresponding to mRNAs induced by amino acid starvation. One 632 bp cDNA identified by the screening was characterized. The mRNA of the ASI (amino acid starvation-induced) gene was induced up to three-fold after 12 hours of incubation in amino acid-free Krebs-Ringer bicarbonate buffer. The relative abundance of several other mRNAs such as actin and glyceraldehyde-3-phosphate dehydrogenase decreased during the same period of starvation. Both cycloheximide and actinomycin D treatment during starvation blocked the

induction of the ASI mRNA. The half-life of the ASI mRNA in the presence of actinomycin D was determined to be approximately 7.5 hours in both the fed and starved conditions. Incubation of cells in amino acid-supplemented medium containing the proline analog L-azetidine-2-carboxylate caused the ASI mRNA to be induced less than 2-fold. The ASI mRNA was present in every rat tissue tested. The corresponding full-length cDNA was sequenced, with no significant homologies to any sequences contained in the GenBank data base. Southern blot data showed the ASI gene to be probably single copy in human, chicken, alligator, and yeast. Southern analysis of several rodent DNAs tested yielded a middle-repetitive pattern, believed to be due to a rodent-specific repetitive element or "retroposon" present in the ASI DNA sequence. The full-length protein coding sequence, obtained by the anchored polymerase chain reaction method, was ligated into a pGEM-9Z plasmid vector and synthetic mRNA was made *in vitro*. When this mRNA was translated in a rabbit reticulocyte *in vitro* translation system, electrophoretic analysis showed the synthesis of an approximately 22 kDa polypeptide, which compares well with the 21.4 kDa predicted weight based on the amino acid composition derived from the cDNA sequence.

CHAPTER 1

INTRODUCTION

Although examples of regulation by substrates are plentiful in prokaryotic systems, there are few well documented systems in mammalian cells or tissues that illustrate regulation at the gene level by substrate availability. The first and foremost example of nutrient regulated transcription is the lac operon of E. coli, elucidated primarily by Jacob and Monod (Pardee et al, 1959). The binding of lactose to a repressor protein releases genetic repression of the lac operon, allowing increased transcription of the lactose permease and B-galactosidase. The protein products allow the cell to respond to changes in lactose availability. When lactose concentrations fall as the cell utilizes the sugar, binding of the repressor protein to the DNA regulatory region is increased, thus re-establishing the repression of the lac operon gene expression. Also in bacteria, depletion of a single amino acid leads to increased transcriptional activity of those genes responsible for the enzymes in the cognate pathway. For example, bacteria adapt to starvation

of L-tryptophan or L-histidine by elevating levels of expression of all the enzymes responsible for the biosynthesis of the appropriate amino acid (Miller and Reznikoff, 1978).

In contrast to the specific regulation by the substrate of the lactose or amino acid operons of E. coli, a more general control mechanism is seen in the yeast Saccharomyces cerevisiae. Expression of many genes encoding enzymes of several different amino acid biosynthetic pathways increases in response to amino acid starvation. The term general control of amino acid biosynthesis, or "gcn", has been used to describe the cross-pathway character of this response. Over 30 enzymes in nine different amino acid biosynthetic pathways have been shown to be regulated by starvation of yeast cells for any one of at least 10 amino acids (Hinnebusch, 1988). The biosynthetic pathways that are regulated by substrate include tryptophan, arginine, histidine, lysine, leucine, and methionine. The response to amino acid starvation in yeast is rapid, with elevated steady-state levels of controlled enzymes being established in less than an hour when, for example, cells are shifted from a rich to a minimal medium (Hinnebusch, 1986). Since the control of the gcn response has been shown to be at the level of transcription, and the genes controlled by the gcn response are unlinked in the yeast genome, the general control system has proved to be a useful model for the study

of coordinate regulation of unlinked genes in a eucaryotic organism. In the last five years, it has become clear that the coordinate regulation of the gcn system depends on a short nucleotide sequence (5'-TGACTC-3') found in the upstream region of each gcn-regulated structural gene (Arndt et al., 1987).

It remains unclear what the exact mechanism is for detecting the amino acid-starved condition in yeast. Depletion of an amino acid pool is sufficient to derepress the expression of amino acid biosynthetic enzymes, but it is not a necessary condition to elicit the general control response. This has been demonstrated in the mutant yeast strain ils1, which exhibits low levels of isoleucyl-transfer RNA (tRNA) synthetase activity along with low levels of isoleucyl-tRNA. With normal cellular concentrations of isoleucine present, this cell line displays elevated expression of enzymes in at least four amino acid biosynthetic pathways that are normally derepressed by isoleucine starvation in wild type cells (Messenguy and Delforge, 1976). This derepression seen in the ils1 strain is blocked by a mutation in the GCN1 gene, a positive regulatory gene that is required for gcn-mediated derepression (Niederberger et al, 1983). This suggests that a reduction in the amount of charged tRNA or the reduced rate of protein synthesis is more likely to be the detected

signal for derepression rather than the depletion of an amino acid pool.

The HIS4 gene is a member of the histidine biosynthetic pathway, and shows a three- to four-fold derepression in response to amino acid starvation. This derepression is completely dependent on the GCN4 gene (Hope and Struhl, 1986). GCN4 encodes a trans-acting transcription factor that is the most positive regulator of transcription in the gcn response. Not only is the increase in transcription of the HIS4 gene dependent on the GCN4 protein, but two-thirds of the basal level of transcription of HIS4 is GCN4 dependent as well. Deletion studies along with DNA sequencing of the 5' end of the HIS4 gene have identified six sets of 12 base pair sites containing the GCN4 binding sequence. Along with those six sites is a single 14 base pair region that is required for normal basal expression of the HIS4 gene (Lucchini et al, 1984). Other well characterized gcn-controlled regulatory regions are contained within the HIS3 and TRP5 genes. The HIS3 gene has 7 sites in the 5' region where GCN4 may bind as well as an additional region necessary for basal expression (Struhl, 1982). The TRP5 regulatory region has been shown to contain two GCN4-associated binding regions along with a single region essential for basal expression (Zalkin and Yanofsky, 1982).

Although GCN4 is known to be the most positive regulator involved in the gcn-mediated response, it should be noted that there are a total of 9 GCN genes involved in the response, and a recessive mutation in any of the nine will block derepression of enzymes regulated by general control. There also exists a set of genes termed GCD, that function to maintain repression under nonstarvation conditions, probably by acting as negative regulators of the GCN4 and other GCN proteins. GCN1, GCN2, and GCN3 serve as positive regulators of GCN4; this regulation is at the level of translation (Mueller et al., 1987). To confirm this, the 5' noncoding sequence of the GCN4 gene was replaced with the transcriptional upstream activation site (UAS) of the GAL1 gene. Although this construct now makes GCN4 transcription dependent on the presence of galactose, GCN4-directed derepression in response to starvation is normal (Mueller et al., 1987). The mechanism of translational control of GCN4 is dependent on four upstream AUG codons. These start codons are followed by one or two sense codons and then an in-frame termination codon. Deletion of these short reading frames in the 5' non-coding region results in constitutive gcn-directed derepression, even though the abundance of the GCN4 mRNA was essentially unchanged by the deletion (Thireos et al., 1984). A heterologous transcript was constructed from the 5' upstream GCN4 region followed by a hybrid GAL1-

lacZ fusion gene. This heterologous gene was found to exhibit the characteristic gcn derepression in response to amino acid starvation (Mueller *et al.*, 1987). Point mutations that disrupted the four AUG codons resulted in constitutive derepression of the gene construct (Mueller and Hinnebusch, 1986). The GCD1 gene is the major factor that interacts with the 5' region of the GCN4 mRNA. The translational control between the four upstream AUG codons, the GCD1 gene, and three other positive regulators GCN1, GCN2, and GCN3 all interact to control the production of GCN4 protein, which in turn controls gcn-mediated derepression.

In contrast to the general control system of yeast that has been worked out in great detail, only a few mammalian systems have been identified that show regulation by metabolites or substrates. One of the inherent difficulties in studying substrate-dependent control in higher organisms is the fact that *in vivo* a change in metabolite concentration can cause changes in enzyme activity via a complex hormonal or neural process rather than by direct transcriptional or translational control by the substrate itself (Morley *et al.*, 1988). Resolution of this problem must be accomplished by the use of *in vitro* studies to corroborate any results seen *in vivo*.

Expression of 3-hydroxyl-3-methylglutaryl coenzyme reductase (HMG CoA reducatase) has been found to be

regulated at the gene level by sterol (Chin et al., 1985). Assays for mRNA abundance and mRNA transcription have shown that the presence of an oxysterol acts to reduce the transcription rate of the HMG CoA reductase gene (Goodridge, 1987). Sequences responsible for mediating the promotion and inhibition of transcription have been identified (Osborn et al., 1985). At least two elements upstream from the transcription start site are necessary for the basal transcription of the HMG CoA reductase gene (Osborn et al., 1987). These two regions are 85 bp and 30 bp upstream from the initiation codon.

Glucose has been shown to have a direct effect on a few genes, the best example being the two glucose-regulated proteins (grp's). One of these proteins is a component of the endoplasmic reticulum and may be serving some function relating to the regulation of glycosylation of proteins in this compartment (Munro and Pelham, 1986). Deprivation of glucose causes a 10- to 20-fold increase in the steady-state levels of mRNA for these proteins (Lin and Lee, 1984).

Pyruvate kinase of liver is a key enzyme in the glycolytic pathway whose activity and mRNA levels in the liver fluctuate according to dietary status. mRNA levels of this gene are controlled by both glucose and insulin in cultures of plated hepatocytes. Increases in glucose concentration from 5mM to 40mM were shown to raise pyruvate

kinase mRNA levels about 20-fold, even while insulin levels remain constant (Decaux et al., 1989).

Deprivation of D-glucose will cause an up-regulation of the facilitative D-glucose transporter in rat brain glial cells (Walker et al., 1988). This regulation is seen both as increased transport activity for D-glucose as well as a 4- to 6-fold increase in the abundance of the glucose transporter mRNA. The induction is complete 6 to 12 hours after the onset of glucose deprivation. The mechanism(s) behind these examples is still unknown.

A variety of metals have also been shown to be able to transcriptionally activate genes. Zinc, cadmium, copper, and mercury all can activate one or more of the metallothionein genes. This induction has been seen in hepatic tissue (McCormick et al., 1981), in the kidney (Swerdel and Cousins, 1982), and the induction has been shown to be due to a transcriptional activation (Durnam and Palmiter, 1981). The 5' regulatory region of the metallothionein-IIA gene has been sequenced and characterized (Karin et al., 1984) and in this case, there are two "metal ion-responsive elements," or MRE's, located about 140 and 40 bp upstream from the translational start site. These sites are binding sites for proteins that mediate the response to metal toxicity, and when these proteins are bound to the MRE, then transcription of the gene is enhanced.

Liver is the major site of gluconeogenesis in the mammalian organism. As a result, transport of gluconeogenic precursors by this tissue is a necessary process and, in fact, is the rate-limiting step for hepatic gluconeogenesis from alanine (Fafournoux et al., 1983). The general properties of transport systems as well as the phenomenon of adaptive regulation (Gazzola et al., 1972) have been investigated. Adaptive regulation is a process by which cells increase their transport rates for certain amino acids when confronted with low extracellular concentrations of these amino acids (Riggs and Pan, 1972; Gazzola et al., 1972). This response is mediated primarily through enhancing the activity of the amino acid transporter System A, which was discovered over 25 years ago in the Ehrlich ascites tumor cell (Oxender and Christensen, 1963). This adaptive response in cultured hepatocytes and hepatoma cells is first seen 60 to 90 minutes after cells are placed in an amino acid-free medium (Kilberg et al., 1985). The translation inhibitor cycloheximide (Handlogten et al., 1982), the transcription inhibitor actinomycin D (Kelley and Potter, 1978), and N-linked glycoprotein inhibitor tunicamycin (Barber et al., 1983) prevent the increased System A transport activity of adaptive regulation. The prevention of stimulated System A transport in adaptive regulation by tunicamycin suggests that System A is a

glycoprotein or, at least, that its activity is dependent on the de novo synthesis of a glycoprotein.

Collectively, the data are consistent with a model of adaptive regulation that proposes that a gene coding for a plasma membrane protein, perhaps the System A carrier itself, is more actively transcribed (derepression), and the resulting mRNA transcripts are translated to make more membrane protein (Handlogten et al., 1982). This hypothesis is supported by kinetic experiments showing that during adaptive regulation the Vmax of System A transport increases rather than a change in Km for the substrate (Gazzola et al., 1972), suggesting an increase in the number of functional transporters. Furthermore, the fact that one can isolate plasma membranes containing stimulated System A activity from amino acid starved cells argues for additional carrier synthesis (Fong et al., 1990). Although there may be other more complicated explanations for the inhibitor and transport results, we believe the simpler working model of increased transcription of the System A gene and production of additional System A carrier protein to be a sound basis for further experimention.

Along with the increase of System A transport seen during amino acid deprivation, there is an adaptive repression of the enhanced transport activity in the presence of amino acid-containing medium (Gazzola et al., 1972). Amino acids may inactivate stimulated System A

activity by either a protein synthesis-dependent (repression) or protein synthesis-independent ("trans-inhibition") mechanism (Kilberg et al., 1985; Bracy et al., 1986). Although there are exceptions (Boerner and Saier, 1985; Englesberg and Moffett, 1986), there are also a number of amino acids, the branched chain and aromatic amino acids as examples, that have no regulatory effect on System A activity when added back to amino acid-starved cells. Other amino acids, such as histidine and glutamate exhibit an inhibitory effect that appears to be due mostly to the protein synthesis-independent mechanism called trans-inhibition (Bracy et al., 1986). It has been found that only the amino acid substrates of System A act to repress activity by the protein synthesis-dependent mechanism (Bracy et al., 1986), with alanine, asparagine, glycine, proline, serine and threonine being the most effective repressors found. This repression is blocked by both cycloheximide and actinomycin D (Kilberg et al., 1985). When adequate levels of amino acids are restored, the excess functional transporters in the plasma membrane are presumably degraded, and the elevated transcription rate slowed.

It is important to recognize that our lab has studied the amino acid-dependent regulation of the System A amino acid transporter, and we are interested in examples of amino acid regulation of metabolic processes, and gene expression in particular. It has been well documented that amino acids

exert a regulatory effect on the catabolism of protein in hepatic tissue (Poso et al., 1982), with seven amino acids able to directly regulate the degradation, and alanine able to co-regulate degradation. As concentrations of the amino acids remain high, catabolism is blocked, but if the plasma concentration of the controlling amino acids drops, then degradation is favored. This phenomenon is seen as control at the metabolic level, very different from what the inhibitor studies suggest is happening with System A.

There exist few reports in the literature regarding the induction of proteins responding specifically to amino acid starvation. Levinson et al., (1980) observed that amino acid starvation of cultured chick embryo cells induced the biosynthesis of four proteins. Two hours after incubation of cells in amino acid-free medium, proteins corresponding to molecular weights of 89, 73, 35, and 27 kDa were induced. Biosynthesis reached steady state within 8 hours, and began to decay after 12 hours of culture in the amino acid-free medium.

The activity of the methionyl-tRNA synthetase component of the multi-enzyme aminoacyl-tRNA synthetase complex has been shown to be increased upon methionine restriction (Lazard et al., 1987). When methionine levels were dropped from 100 μM to 1 μM in the culture medium of Chinese Hamster ovary cells, a two-fold induction in enzyme activity occurred. Antibodies specific to the methionyl-tRNA

synthetase were used to confirm that the increase in enzyme activity was due to an increased amount of the enzyme. At this time, it is not clear if this induction was transcriptional or post-transcriptional.

Lacking strong examples of regulation by amino acids, our laboratory has made it a goal to investigate the amino acid-dependent regulation of System A, as well as the cellular response to amino acid deprivation in general. To identify proteins that might be up-regulated by amino acid starvation, our lab has used radiolabelling and subsequent two-dimensional gel analysis of rat liver proteins, and more specifically, rat liver membrane proteins to identify proteins whose biosynthesis is enhanced by amino acid starvation, as we predict for the System A protein (Chiles and Kilberg, 1987). The results of one such an experiment are illustrated in Figures 1-1a and 1-1b, which present two-dimensional gel patterns of total cell protein from starved and fed cells. The gel pattern of cellular proteins are nearly identical at 6 hours of amino acid starvation. Previous experiments have identified rat liver membrane proteins of 66 and 73 kDa that were induced 2- to 3-fold by amino acid deprivation (Chiles and Kilberg, 1987).

A second experiment, shown in Figure 1-2, illustrates proteins made <u>in vitro</u> from mRNA purified from Fao hepatoma cells that had been amino acid-fed or starved for three hours. There is very little suggestion of any significant

differences in mRNA abundances in the time scale that our model for System A suggests. The results from Figures 1-1 and 1-2 clearly indicate that there are few proteins induced by starvation that may be seen by this type of gel analysis, although it is known that only the most abundant cell proteins are visible by these types of analyses. The purpose of this project is to identify an mRNA and its corresponding gene that is up-regulated by amino acid starvation in the Fao hepatoma cell line.

(a)

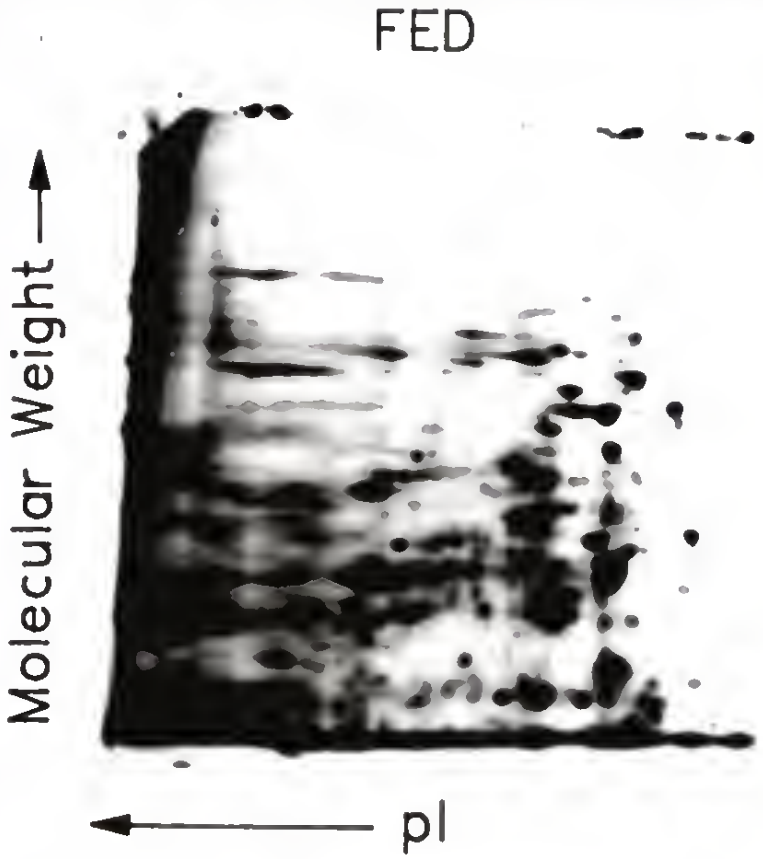

Figure 1-1. Autoradiogram of Fao cellular protein obtained from cells cultured for 6 hours with or without amino acids following Two-Dimensional Polyacrylamide Gel Electrophoresis. Cells were incubated in the presence of S^{35}-Methionine, total protein isolated and separated by pI in the horizontal direction and by molecular weight in the vertical direction. The gels were subsequently dried and used for autoradiography (protein gel methods presented in chapter 4). Figure 1-1a shows cells incubated in the presence of amino acids (MEM) while Figure 1-1b (next page) shows cells incubated in the absence of amino acids (NaKRB).

(b)

Figure 1-1. continued.

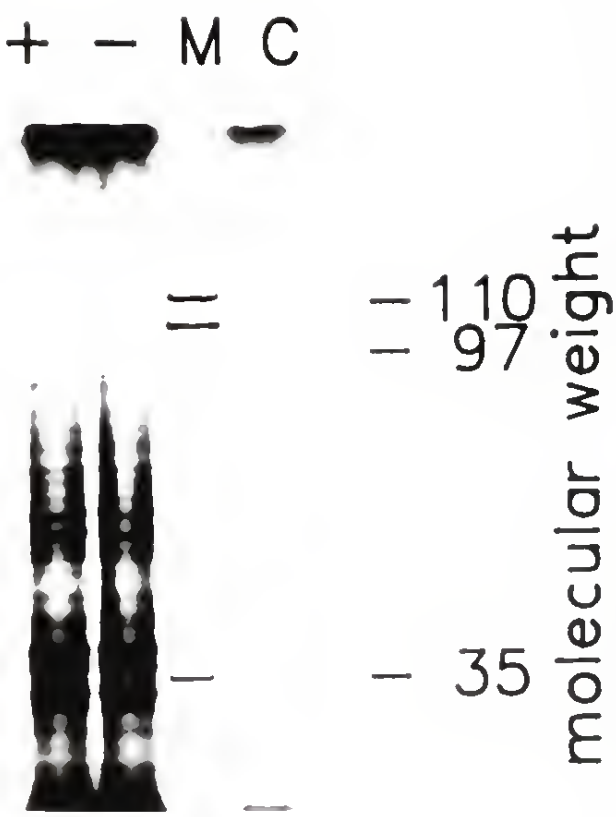

Figure 1-2. Autoradiograph of <u>in vitro</u> translation products following One-Dimensional SDS-PAGE. Poly(A)$^+$ mRNA was purified from Fao cells amino acid-starved and -fed for three hours. Five micrograms of mRNA was then translated in the presence of H^3-Leucine using the rabbit reticulocyte translation system (<u>in vitro</u> translation methods presented in chapter 4). Proteins were size fractionated by electrophoresis, the gel was then dried and used for fluorography. Lanes on gel: 1 (+), amino acid-fed cells; 2 (-), amino acid-starved cells; 3 (M), Brome Mosaic Virus mRNA, used as molecular weight markers; 4 (C), Control -- no mRNA added to the translation mix.

CHAPTER 2

IDENTIFICATION OF mRNAs IN Fao RAT HEPATOMA CELLS INDUCED BY AMINO ACID STARVATION

Introduction

To gain insight into the mechanisms by which higher eucaryotic systems are regulated by availability of substrate, we proposed to identify a cDNA clone corresponding to a gene that is amino acid regulated by using the methods of subtractive library production and differential hybridization (Figures 2-1 and 2-2). The procedure of differential hybridization uses cDNA probes derived from two different populations of mRNA molecules in contrast to other methods that derive probes from a single gene or gene product such as specific DNA segments. This differential, or "plus/minus" procedure is designed to detect cDNA clones derived from mRNAs which are present ("plus") in one condition and absent or reduced ("minus") in a second condition. The method is one of the less common methods of identifying or cloning genes, compared to the screening of cDNA expression libraries with antibodies, or screening libraries with DNA probes, but nonetheless, hundreds of genes have been identified by this method (Sargent, 1987).

One of the first examples of differential hybridization being used was from Williams and Lloyd (1979) in their study of the slime mold, D. discoideum. A cDNA library, or "clone bank", as they referred to it, was created from the organism's mRNA, purified at the eighth hour of development in liquid culture. The clone bank was screened using mRNA obtained from the eighth hour ("plus") of development and the beginning of the culture period, or zero hours of development ("minus"), end-labelling the RNA with [^{32}P]-ATP to make it a radiolabelled probe. A number of clones corresponding to high and medium abundance mRNAs were obtained for which the relative level changed during the first nine hours of development.

Dworkin and Dawid (1980a, 1980b) produced a similar study examining changes in mRNA abundance during embryonic development of the African clawed frog, Xenopus laevis. In this work, several cDNA libraries were constructed from a variety of stages in development, and they were screened by radiolabelled first-strand cDNA using the method of colony hybridization (Grunstein and Hogness, 1975). Dworkin and Dawid identified many different clones showing a differential pattern of expression both increasing and decreasing throughout development.

Many other examples of differential hybridization as a method for identifying regulated genes exist in the

literature. Some of these include clones from Saccharomyces inducible by galactose (St. John and Davis, 1979); identification of the T-cell receptor (Saito et al., 1984); mRNAs under the influence of hormones such as triiodothyronine (Magnuson et al., 1985), estradiol (Masiakowski et al., 1982), and progesterone (Misrahi et al., 1987). Other studies have identified mRNAs that are sex-specific (Zurita et al., 1987), under the control of the circadian clock in Neurospora (Loros et al., 1989), DNA damage-inducible (Fornace et al., 1988), and transiently induced during rat liver regeneration (Sobczak et al., 1987).

From these examples, it is clear that if specific differences exist between two mRNA pools, then identification of those differentially expressed mRNAs is possible if there is a significant difference, and the mRNA is not unusually rare. We believe that cells cultured in amino acid-free and amino acid-supplemented media have specific differences in their mRNA pools, one of the differences between the two pools being a class of mRNAs for which the abundance is increased when cells are cultured in amino acid-free media. On the basis of inhibitor studies, we believe that the System A gene in rat liver and hepatoma cells is a member of this class of mRNAs (Kilberg, 1986).

To enhance the probability of finding clones for amino acid regulated genes, of which the total number and abundance is unknown, two things may be done. First, the source of the mRNA should be a cell type or cell condition that maximizes the abundance of the class of mRNA in question. To this end, the Fao hepatoma cell line was chosen because it is relatively easy to grow in cell culture, and consistently shows a strong adaptive regulation response. That is, System A transport activity in the Fao cell is reproducibly three- to four-fold higher in the amino acid starved condition compared to the cells supplemented with amino acids. Furthermore, the Fao cell line does not secrete appreciable amounts of albumin compared to hepatocytes, suggesting that the mRNA abundance of albumin is greatly reduced in the Fao as well, thus eliminating the most abundant mRNA of the liver cell. Performing the experiment in cell culture rather than *in vivo* allowed us to separate the condition of starvation from hormonal changes that would occur due to dietary differences in the rat. As there was no mRNA known to be induced by amino acid starvation in any mammalian system, we followed the enhanced activity of the System A transporter as our indicator that the cells were entering adaptive regulation. mRNAs for which abundances increase during adaptive regulation are assumed to increase along with the increase in System A-

mediated transport. This correlation between mRNA content and related protein abundance has been documented in the hormonally regulated gluconeogenic enzyme, PEPCK (Beale et al., 1982). We chose to make a cDNA library using mRNA purified from the amino acid-starved Fao cells displaying the adaptive regulation response.

To further increase the probability of finding an mRNA up-regulated by starvation, a second library was made with amino acid-starved Fao cells as the source for mRNA, but in this case, subtractive hybridization was used to increase the effective abundance of induced clones in the library. Subtraction is made possible by the fact that mRNA populations can be readily hybridized to completion using homologous cDNA (Sargent, 1987). If "induced" mRNA from starved cells is used to generate first-strand cDNA templates, then they may be hybridized to an excess amount of "uninduced" mRNA purified from fed cells. Sequences in common readily hybridize to one another, but cDNA sequences enhanced in the induced condition will have fewer homologous counterparts from the uninduced condition, and are therefore less likely to hybridize. These molecules that remain single stranded represent a population of cDNAs that are more likely to contain induced sequences and can then be used to generate a "subtracted" cDNA library. Such a subtracted library was made and was screened using

differential hybridization to identify amino acid regulated clones.

In either case, from the induced mRNA of the starved Fao cells, or the subtracted cDNA, cDNA libraries were made in the phage vector lambda gt11. These libraries are referred to as the "induced" library, and the "subtracted" library, respectively. Lambda gt11 was engineered as a cDNA vector that would also express the gene product of the cloned cDNA as a fusion protein, that is, a chimeric protein consisting of the polypeptide corresponding to the cloned cDNA linked to the E. coli B-galactosidase protein, whose gene was inserted into the vector (Huynh et al., 1985). This construction would allow the expression libraries to be utilized should antibodies be produced to amino acid-starvation induced proteins. Once the libraries were created, they were then screened by the "plus/minus" method, and induced cDNA clones identified.

Materials and Methods

Cell culture

Fao Hepatoma cells were grown in Modified Eagle's Medium (MEM) (pH 7.4) supplemented with 24 mM $NaHCO_3$, 2.5 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 28.5 µg/ml gentamicin, and 6% fetal bovine serum. Incubation was at 37°C with 5% CO_2 added to the atmosphere. Cells were grown to near confluence, prior to preparation of RNA,

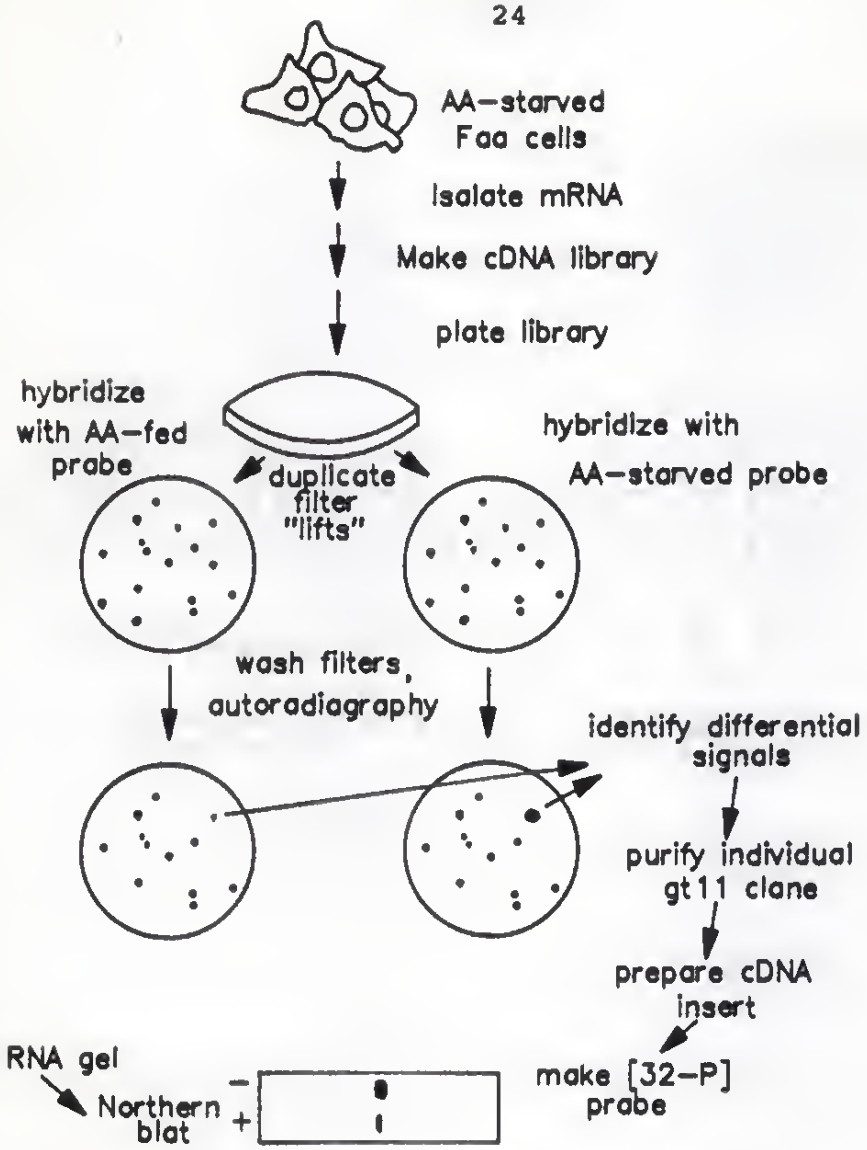

Figure 2-1. Diagram illustrating the general protocol involved in identification of an mRNA induced by amino acid starvation. Cartoon illustrates the preparation of a cDNA library from amino acid-starved Fao cells, the library is plated on an agar surface and duplicate nitrocellulose lifts are prepared from the agar surface. The two lifts are hybridized separately with cDNA probes made from mRNA prepared from fed and starved cells. Differential hybridization signals are identified, and the cDNA insert is purified from the library clone responsible for the differential pattern. The induced status of the mRNA is confirmed using the purified cDNA as a probe on a Northern blot containing lanes of mRNA prepared from fed and starved cells.

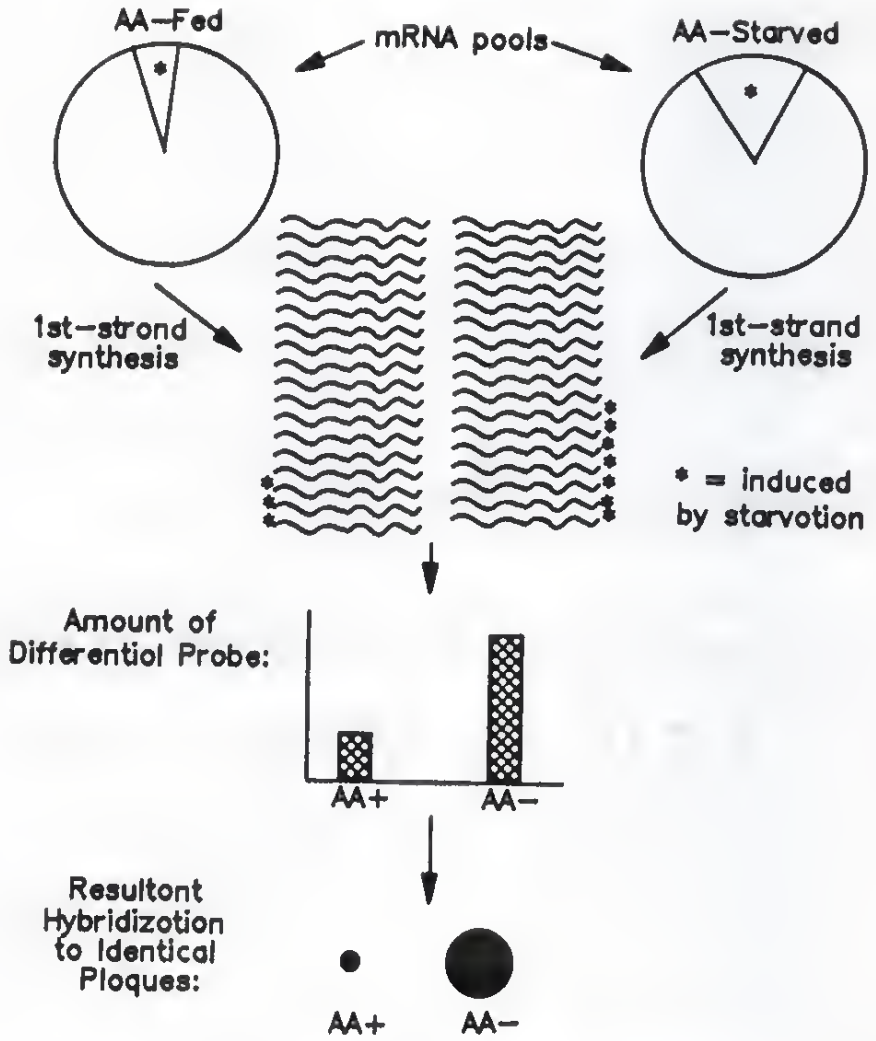

Figure 2-2. Diagram illustrating principle of differential hybridization. mRNA pools, represented by "pie" graphs, depict a class of mRNAs induced by starvation (marked with an asterisk). That difference in abundance is reflected in the population of cDNA molecules (curved lines) made from the two populations of mRNA. cDNAs corresponding to the induced class are marked with asterisks. For a given quantity of total cDNA made, there will be more cDNA from the induced class in the starved case compared to the fed case (bar graph). This difference will be reflected after hybridization to equal amounts of DNA fixed to filters, as seen by autoradiography.

transport assays, or passage to new flasks for continued culture.

Amino acid uptake by Fao hepatoma cells

To ascertain that cells in culture have responded appropriately to amino acid starvation prior to preparation of RNA, cells were assayed for System A transport activity. Along with 150 mm tissue culture plates prepared for RNA isolation, four extra plates of cells were prepared for this assay. On the day of harvest, the cell media was removed and replaced with sterile Na^+-containing Krebs-Ringer bicarbonate (NaKRB) buffer supplemented with antibiotics. One half of the dishes received buffer supplemented with 3 mM of each of the six amino acids (alanine, asparagine, glycine, proline, serine, and threonine) known to repress System A activity to a basal level (Bracy et al., 1986). After three hours of incubation in these media, cells in the dishes were harvested for RNA purification by the method of Chomczynski and Sacchi (1987). Concurrently, the four extra dishes were used to test the System A activity in the amino acid-starved or amino-acid supplemented dishes.

Amino acid transport was measured by the method of Gazzola et al., (1981), except that 15 cm dishes were used instead of cluster trays. Briefly, cells were incubated in the presence of the radiolabelled amino acid AIB (200 μM) with a Na^+-containing or Na^+-free buffer. Na^+-dependent

transport for 2 minutes at 37°C was taken as the difference between the uptake rate in the two buffers. Following the transport assays, the cellular protein in the dishes was precipitated with the addition of 10 ml of 10% trichloroacetic acid (TCA). After incubation for 1 hour at 4°C, 0.2 ml of the extract was placed in a scintillation vial for determination of the radioactivity, whereas the remainder of the extract is discarded. The precipitated protein in each dish was solubilized with 5 ml of 0.2 M NaOH containing 0.2% SDS and then measured by a modification of the Lowry procedure (Kilberg et al., 1983).

Handling of DNA and RNA

Methods used were generally as described in Maniatis (1982). DNA and RNA concentrations were measured at 260 nm, using 50 $\mu g/ml/A_{260}$ and 42 $\mu g/ml/A_{260}$, respectively. Ethanol precipitation of DNA was at -20°C for at least one hour in the presence of 2.5 M ammonium acetate, pH 7.5, or 0.3 M sodium acetate, pH 5.5, and two volumes of ethanol or one volume of isopropanol. Precipitates were collected by centrifugation, large volumes at 10,000g for at least 10 minutes, small volumes (in microfuge tubes) at 14,000g for at least 10 minutes. RNA was precipitated for at least one hour in the presence of 0.2 M sodium acetate, pH 4.0, and two volumes of 100% ethanol or one volume isopropanol.

Precipitates were collected by centrifugation as described for DNA.

Phenol/chloroform extraction refers to the deproteination of a nucleic acid solution by emulsification with 1 volume of phenol, followed by re-emulsification with one volume of 1:1 (v/v) phenol/chloroform, and lastly one volume of chloroform alone. Aqueous phases at each step were recovered by centrifugation.

All solutions and materials that came in contact with RNA were autoclaved or filter sterilized. Solutions, test tubes, pipet tips, and other materials were pretreated overnight with a solution of 0.01% diethylpyrocarbonate (DEPC) prior to autoclaving to inactivate RNases.

RNA isolation

Total cellular RNA was prepared using the "Single-Step Extraction" method of Chomczynski and Sacchi (1987). Cells (or tissue) were solubilized in "Solution D", which contains 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, and 0.5% sarcosyl. For a 150 mm tissue culture dish, 6 ml of solution D were added, and for tissues, about 5 ml were used per gram of tissue. After lysis of tissue culture cells or homogenization of tissue in Solution D, the solution is acidified by adding 0.1 volume of 2 M sodium acetate, pH 4.0. An equal volume of water-saturated phenol is added and mixed by inversion followed by the addition of

0.2 volumes of chloroform. After vigorous mixing for 10 seconds, the mixture is incubated on ice for 15 minutes followed by a centrifugation for 20 minutes at 10,000g. The aqueous phase containing the RNA is collected and the RNA is precipitated by the addition of an equal volume of ice-cold isopropanol followed by a 1 hour incubation at -20°C. The RNA is collected with a 10 minute centrifugation at 10,000g. The pelleted RNA is resuspended in 0.3 volumes of the original amount of Solution D. We then added the extra step of extracting this volume with an equal amount of 1:1 (v/v) water-saturated phenol/chloroform to further remove any protein contaminants. The RNA was again precipitated with an equal volume of isopropanol, and the RNA pelleted as before. After rinsing the pellet with 80% ethanol, the RNA was air-dried and resuspended in DEPC-treated water. The absorbance of the RNA solution was then determined at 260 and 280 nm to determine RNA concentration and the relative abundance of nucleic acid and protein. This 260:280 ratio should be about 2.0 as an indication that the RNA is reasonably pure. RNA obtained by these methods was size fractionated by formaldehyde/agarose gel electrophoresis (Davis et al., 1986), stained with ethidium bromide, and densitometrically scanned to quantitate relative amounts of 18S and 28S ribosomal RNAs (complete descriptions of RNA electrophoresis and densitometry are in Chapter 3 methods).

The ribosomal 28S:18S ratio should be about 2:1, indicating that little degradation of RNA by ribonucleases has occurred, and the RNA is of good quality to use for reverse transcription (Han et al., 1987).

Poly(A)$^+$mRNA isolation

Poly(A)$^+$mRNA was separated from other cellular RNA using oligo-(dT) chromatography, essentially following the method of Aviv and Leder (1972). Oligo-(dT) cellulose (Sigma) columns were prepared by first washing 0.25 g oligo-(dT) cellulose in a sterile plastic 12 ml tube with DEPC-treated water. After hydration and gentle mixing, the matrix was allowed to sediment, and the fines were removed with the supernatant by pipetting. The matrix was then resuspended in fresh DEPC-treated water and the slurry added to a sterile disposable plastic 10 ml chromatography column (BioRad no. 731-1550). After the matrix settled, it was equilibrated with at least 10 ml of RNA loading buffer (RNA loading buffer is 0.5 M NaCl, 10 mM Tris, pH 7.4). Total RNA samples, dissolved in water and at concentrations at or less than 1 mg/ml, were mixed with an equal volume of 2X loading buffer, heated at 65°C for 3 minutes and immediately cooled on ice. The solution was then poured over the column, allowed to flow through, and poly(A)$^-$ RNA washed through using 10 ml of loading buffer. The poly(A)$^+$ mRNA was eluted from the column using DEPC-treated water, with

the mRNA typically contained entirely within the first 2 ml of eluent. The eluent was made 0.3 M in sodium acetate, pH 4.0, and precipitated overnight with 2.5 volumes of ethanol. The RNA was then recovered by centrifugation. After one round of purification, the poly(A)$^+$mRNA obtained was fractionated by formaldehyde-agarose gel electrophoresis and stained with ethidium bromide and it was determined that the samples still contained considerable amounts of ribosomal RNA. Therefore, a second round of selection was always done to purify the poly(A)$^+$mRNA to an acceptable level.

cDNA synthesis

To make cDNA for cloning into the bacteriophage vectors lambda gt11, we followed Gubler and Hoffman (1983) as modified by Davis *et al*. (1986) (Figure 2-3). First strand cDNA was synthesized from 10 μg of poly(A)$^+$mRNA using 10 μg of oligo(dT) primer and 40 units of AMV reverse transcriptase (Seikagaiku) in 90 mM Tris, pH 8.7, 130 mM KCl, 9 mM $MgCl_2$, the four dNTPs at 1 mM each, RNAsin (Promega) at 1 unit/μl, and actinomycin D at 36 pg/μl. Fifty microcuries of α-[^{32}P]-dCTP (3000 Ci/mmol) were included to quantitate the reaction yield and to visualize the sizes of the products by autoradiography following alkaline agarose gel electrophoresis (Maniatis, 1982). There was no attempt made to quantitate the yield at successive steps, but recovery of labelled cDNA was always

detected with a Geiger counter every time a precipitation was done. It was found that at least 95% of the counts were always present in the pelleted cDNA. After phenol/chloroform extraction and precipitation using ammonium acetate and ethanol, the first-strand cDNA-mRNA duplex was dissolved in 50 μl of TE buffer (TE buffer is 10 mM Tris, pH 7.5, 1 mM EDTA). Two microliters of this volume were removed and used for quantitation by liquid scintillation spectrometry and electrophoresis. The remainder was added to 50 μl of 2X second-strand buffer (2X buffer is 40 mM Tris, pH 7.4, 1 mM MgCl2, 2 mM ammonium sulfate, 200 mM KCl, BSA at 100 μg/ml and the four dNTPs at 80 μM each). Two units of RNaseH and 11.5 units of DNA Polymerase I were added, and this reaction was incubated for 1 hour at 12°C, followed by one hour at 22°C. This reaction mixture was extracted two times with chloroform, and the cDNA was then precipitated twice with ammonium acetate and ethanol to remove the unincorporated dNTPs from the cDNA. Methylation of the cDNA at internal EcoRI sites was done using 100 units of EcoR I methylase and 15 μM S-adenosyl methionine in 100 mM Tris, 5 mM EDTA buffer (pH 8.0) for 20 minutes at 37°C. Following a 10 minute incubation at 65°C to inactivate the enzyme, the ends of the cDNA were blunted or "polished" using 15 units of T4 DNA polymerase in the presence of 100 μM dNTPs and 12 mM $MgCl_2$ in the same

Tris/EDTA buffer used for the methylase reaction. This mixture was incubated for 15 min at 37°C, after which the cDNA was subjected to an organic extraction followed by ammonium acetate and ethanol precipitation. The cDNA, resuspended in 10 mM Tris, pH 8.0, had phosphorylated DNA linkers containing the EcoR I-specific sequence added to the polished ends using 1,600 units of T4 ligase. The ligation was allowed to incubate overnight at 15°C. The cDNA was then digested with the restriction endonuclease EcoR I to generate cohesive EcoR I ends for cloning into the EcoR I site of lambda gt11 phage. The digested cDNA was size-fractionated on a 5% polyacrylamide gel in 50 mM Tris, 50 mM boric acid, 1 mM EDTA (TBE) buffer along with a parallel lane of DNA size markers. All cDNA over 500 bp was excised from the gel, electroeluted in TBE buffer, and then concentrated using an Elutip-d mini column following the protocol supplied by the manufacturer (Schleicher and Schuell). Briefly, the small prepacked plastic column allows DNA to be bound to the matrix in a low salt buffer, the column is rinsed, then DNA eluted in a volume of 400 μl with a high salt buffer containing 1 M NaCl. The cDNA was then ethanol precipitated and resuspended in TE buffer. The final yield of cDNA was estimated based on the specific radioactivity of the first strand cDNA synthesized. Second strand reactions are known to be nearly 100% efficient and

need not be monitored as closely as the first strand synthesis.

Library construction

The cDNA, made as described above, was ligated into dephosphorylated arms of the vector lambda gt11 (Promega) and packaged *in vitro* using the "Packagene" *in vitro* packaging extract (Young and Davis, 1983) from Promega Biotec, following the protocol supplied by the manufacturer. Briefly, the cDNA inserts were ligated to the dephosphorylated arms of the vector using T4 DNA ligase at 15°C for four hours, followed by incubation with the packaging extract for 2 hours at room temperature to create infective phage. An aliquot of the packaging reaction was taken and plated on *E. coli* host strain Y1088 to quantitate the total number of plaque forming units, or "p.f.u" in the mixture.

Subtractive hybridization

Total cytoplasmic RNA was isolated from amino acid-starved and amino acid-fed cells, poly(A)$^+$mRNA selected by oligo-(dT) chromatography, and first strand cDNA was made from the poly(A)$^+$mRNA from amino acid-starved cells using AMV reverse transcriptase as described previously to obtain a mRNA/cDNA duplex. The original mRNA template was then hydrolyzed by adding NaOH to 0.4 M and heating at 50°C for 1 hour followed by 65°C for 15 minutes. This mixture was then

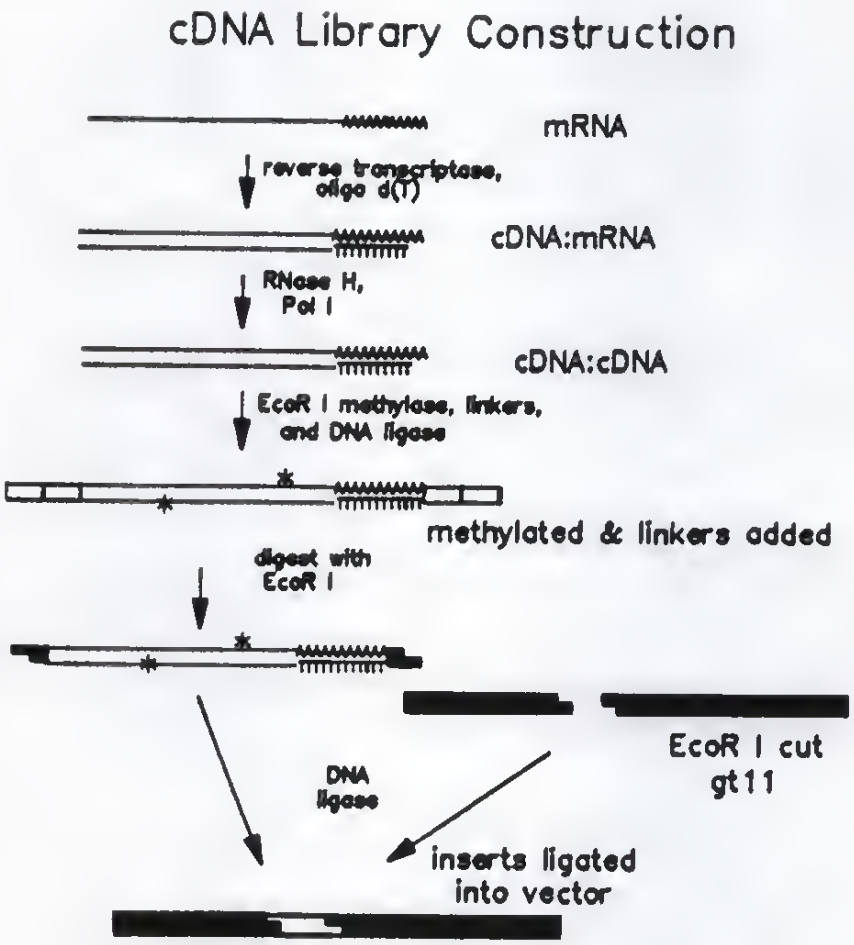

Figure 2-3. cDNA library construction. Outline of cDNA construction from poly(A)$^+$ mRNA using the method of Gubler and Hoffman. mRNA is reverse transcribed from mRNA using an oligo(dT) primer and reverse transcriptase. A cDNA duplex is made using RNaseH and DNA polymerase I. The cDNA was tailed with EcoR I linkers, and ligated into the phage vector lambda gt11.

neutralized with HCl, phenol-chloroform extracted, precipitated by adding 0.4 volumes of 6M ammonium acetate and 3 volumes of ethanol and then incubating in a dry ice/ethanol bath for 45 minutes. The cDNA was collected by centrifugation at 13,000g for 30 minutes. A TCA precipitation analysis of the cDNA quantitated the single-stranded cDNA made from the 10 μg of starting mRNA. The first-strand product was added to 20 μg of mRNA derived from amino acid-fed Fao cells, and lyophilized to dryness. Six microliters of hybridization buffer (0.5 M sodium phosphate, pH 6.5; 0.1% SDS; 5 mM EDTA) were added. The pellet was resuspended and hybridized under mineral oil for 41 hours at 65°C, to a R_0t value of 1500, where R_0t is the product of RNA in moles of nucleotide/liter and time of hybridization in seconds (Timberlake, 1980; Rowekamp and Firtel, 1980). Unhybridized single-stranded cDNAs were separated from cDNA/RNA hybrids by passage over a 15 cm long water jacketed chromatography column containing approximately 10 ml of hydrated DNA-Grade Bio-Gel HTP hydroxyapatite (BioRad). The hybridization mixture was diluted to 0.01 M phosphate, the mixture was then passed over a hydroxyapatite column equilibrated to 0.01 M phosphate, pH 6.5, and kept at 65°C, followed by elution with 0.12 M phosphate buffer, pH 6.5, containing 0.1% SDS with a continuous flow rate of approximately 0.5 ml/minute, to selectively elute the

single-stranded cDNA from double-stranded hybrids which remain bound to the column (Bernardi, 1965) (Figure 2-4). The double-stranded molecules were removed from the column by an elution with 0.5 M phosphate. The single-stranded cDNA was collected, and was precipitated with ammonium acetate and ethanol, resuspended in water, then phenol-chloroform extracted, and precipitated. The resulting pellet was air-dried, then dissolved in 40 μl of sterile water. To this was added 30 μl of random primer extension buffer (0.67 M HEPES, 0.17 M Tris, 17 mM $MgCl_2$, 33 mM ß-mercaptoethanol, 1.33 mg/ml BSA, 18 OD_{260} units/ml $pd(N)_6$ (random deoxynucleotide hexamers), pH 6.8), 5 μl of 1 mM each dATP, dCTP, DGTP, and dTTP, 90 ng oligo-(dT) and 30 units of the Klenow Fragment of DNA Polymerase. This mixture was allowed to incubate at room temperature for 18 hours, and was then subjected to an organic extraction and precipitation as before. The resultant double-stranded cDNA was then methylated at EcoR I sites, ends polished with T4 polymerase, linkers added and digested as described previously. The tailed DNA was size-fractionated on a 5% polyacrylamide gel and a section was cut from the gel representing fragments 500 bp and greater. The gel slice was electroeluted in 0.2X TBE, and the cDNA purified from the buffer using an elutip-d column.

Differential screening

We essentially followed the protocols of Maniatis et al. (1982) for phage library screening. Phage and host bacteria E. coli Y1088 were cultured in LB media, plates were 1.5% agarose and top agarose was 0.8% agarose. Appropriate numbers of infective phage were mixed with 0.2 ml or 0.5 ml (for 100 mm or 150 mm plates, respectively) of host cells and incubated at 37°C for 20 minutes. These cells were then mixed with molten (50°C) top agar; this mixture then was poured over the surface of a 1.5% agarose LB plate. After the top agarose hardened, plates were incubated for 12 to 16 hours at 37°C. After chilling the plates for one hour at 4°C, nitrocellulose filters were placed on the plate until completely wetted, and then carefully removed. Duplicate filters were laid on the plate until wet, then allowed to sit for an additional two minutes to equalize the amount of phage adhering to the two filters. Filters were then treated as follows: air dried, laid on a piece of 3 mm Whatman filter paper soaked with 0.1 M NaOH and 1.5 M NaCl for 2 minutes, and then incubated for 3 minute on a similar Whatman filter soaked with a solution containing 0.2 M Tris, pH 7.5; 1 M NaCl; 0.3 M sodium citrate, pH 7.0; 0.26 M potassium phosphate; 2 mM EDTA. Filters were again air-dried, then baked at 80°C for 2.5 hours in a vacuum oven to fix the denatured phage DNA to the

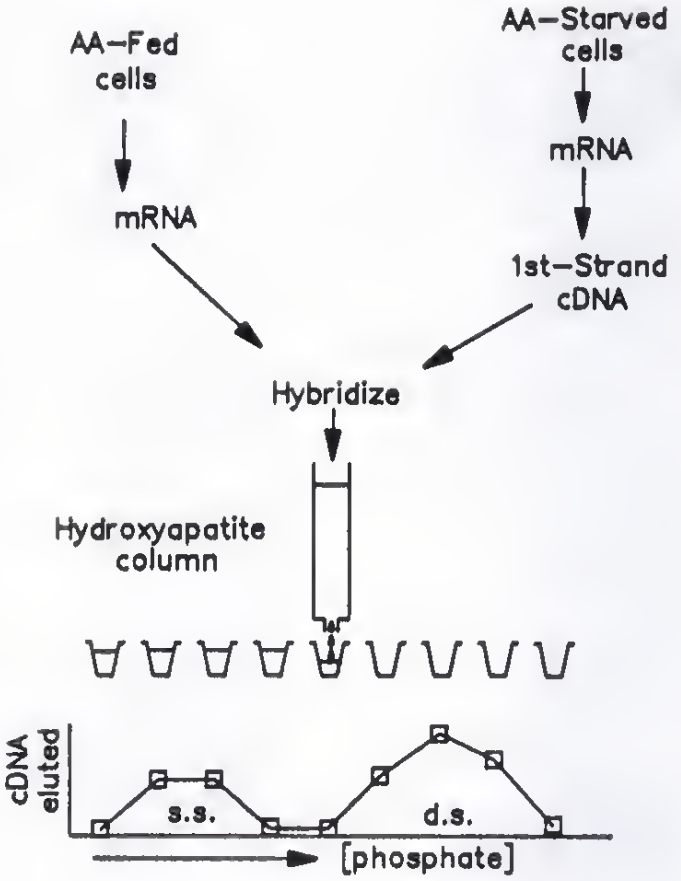

Figure 2-4. Hydroxyapatite chromatographic subtraction of single-stranded cDNA. Protocol outlined here was used to isolate a fraction of cDNA from amino acid-starved Fao cells enriched for induced sequences. First-strand cDNA from starved cells was hybridized to an excess of mRNA from fed cells. After hybridization, the induced, or single stranded cDNAs were collected by passage over a hydroxyapatite column. A hypothetical elution profile is shown. This cDNA was primed with oligo-(dT) and random deoxynucleotide hexamers, made double stranded with DNA polymerase, and then further prepared as illustrated in Figure 2-3.

filters. Pairs of duplicate filters were hybridized with "plus" or "minus" probes as outlined below.

Hybridizations

For differential hybridization procedures between cDNA radiolabelled probes and nucleic acids affixed to nitrocellulose paper, we followed hybridization procedures described by Davis et al., (1986). Briefly, filters prepared as described above were prehybridized in 4X SSC (1X SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7), 0.02 M Tris, 0.02 M polyvinylpyrrolidone, 0.02 M bovine serum albumin, 0.02 M Ficoll, 20 μg/ml sheared salmon sperm DNA, and 10% dextran sulfate. Following prehybridization of at least one hour at 42°C, radiolabelled first-strand cDNA probe was added at 1 x 10^7 cpm/ml. First-strand cDNA probe was made using the first-strand reaction of cDNA synthesis, except that the sole source of dCTP in the reaction mix was 250 μCi of 3000 Ci/mmol α-^{32}P dCTP. Hybridizations were performed at 42°C for 48 hours. After hybridization, blots were washed two times in 2X SSC for 5 minutes at room temperature, two times in 2X SSC, 1% SDS at 65°C for 30 minutes, and then finally two times in 1X SSC for 30 minutes at 65°C. Blots were dried to dampness on filter paper, wrapped in plastic wrap, and used for autoradiography with Kodak XAR-5 film at -70°C.

DNA purification from phage

cDNA inserts from phage were purified as outlined below. Individual isolates of phage were mixed with E. coli strain Y1088 and 0.4%, 50°C molten agarose, and then poured onto a 1.5% agarose plate (for a 15 cm plate, 100,000 p.f.u. of phage were mixed with 150 µl of a suspension of Y1088 concentrated 5-fold from an overnight liquid culture by sedimentation and resuspension in 0.2 volumes of sterile SM buffer). The plate was incubated overnight at 37°C to complete lysis. The top agar containing the phage was collected by scraping, added to 5 ml of SM buffer (SM buffer is 100 mM sodium chloride, 50 mM Tris, 8 mM magnesium sulfate, 0.002% gelatin), and then mixed by rocking for one hour at room temperature. This mixture was centrifuged for 10 minutes at 10,000g to separate the agarose from phage, with the resulting supernatant centrifuged a second time to insure that all traces of agarose have been removed. Following an incubation at 37°C for one hour with 10 µg/ml RNase I and 2 µg/ml DNaseI, whole phage are precipitated by addition of 0.2 volumes of 20% polyethylene glycol (PEG-8000, Sigma), 2.5 M NaCl, mixed, then incubated on ice for 1 hour. Following centrifugation at 10,000g for 20 minutes, the pellet is saved, dried, and resuspended in 0.5 ml of SM buffer. Five microliters of 10% SDS and 5 µl of 0.5 M EDTA are added to this mixture to lyse the phage and prevent

degradation of phage DNA by DNase. The mixture is then subjected to a phenol/chloroform extraction as described previously followed by ethanol precipitation.

Results

We began by producing two lambda gt11 libraries, the first made from total poly(A)$^+$ mRNA isolated from amino acid-starved, or induced Fao hepatoma cells. We refer to this library as the "induced" library. A second library was made from cDNA again derived from induced Fao cells, however, uninduced sequences were subtracted away prior to final library construction. We refer to this as the "subtracted" library.

The induced library was derived from 10 μg of poly(A)+ mRNA prepared from 3 hour amino acid-starved Fao cells induced 3.2-fold for System A transport. Transport assays performed on the batch of cells used for mRNA purification found the sodium-dependent transport of AIB in the Fao cells to be 1645 and 510 pmol AIB/mg protein/minute for the starved and fed cells, respectively. TCA precipitation analysis determined that 0.9 μg of first-strand cDNA was made from the 10 μg of mRNA; this 9% yield is typical of first-strand reactions (Gubler and Hoffman, 1983). From the 0.9 μg of ss cDNA, about 1.8 μg of ds cDNA was made. Following complete processing and purification of the cDNA, we recovered 600 ng of EcoR I tailed, cDNA product. As 1 ng

of DNA represents the equivalent of about 10^9 cDNA inserts averaging 1 kb each, it was clear that there was more than enough cDNA to prepare a library. Typical commercially prepared libraries contain 500,000 to 5 X 10^6 recombinant inserts (e.g. Clontech Laboratories, Palo Alto, CA.).

The cDNA inserts were then ligated to commercially prepared dephosphorylated gt11 arms (Promega) and packaged *in vitro* as described previously. Five different packaging mixes resulted in total numbers of p.f.u. ranging from 60,000 to 437,000. The total for the five mixes was 1.3 x 10^6, which compares favorably to commercially prepared libraries. The collection of 1.3 X 10^6 infective gt11 phage is what is referred to as the induced library. This library was then amplified using *E. coli* Y1090, following the procedure outlined by Davis *et al*. (1986). The amplified lysate contained 10^9 p.f.u./ml, and there was a total volume of 6 ml.

The subtractive library was made from 10 μg of poly(A)$^+$mRNA, and again, the mRNA used was a mixture of two samples. One 5 μg aliquot came from a cell preparation described previously, in which System A transport was 3.2-fold induced by starvation, and the second 5 μg from cells displaying an approximately 17-fold induction (593 vs. 34 pmol AIB/mg protein/minute starved vs fed, respectively. First-strand cDNA was made as described previously using an

oligo-(dT) primer and AMV reverse transcriptase to extend the cDNA product. Approximately 0.6 μg of cDNA was made from the mRNA, which was an acceptable yield for the first-strand reaction (Davis et al., 1986). The cDNA/mRNA duplex was then subjected to alkaline hydrolysis of the RNA as described previously. The single-stranded cDNA was then hybridized to a thirty-fold excess of non-induced mRNA, derived from amino acid-fed Fao cells (System A transport 34 pmol AIB/mg protein/minute). After hybridization to a $R_o t$ of 1500, the single-stranded fraction, representing induced sequences was purified by hydroxyapatite chromatography as described. Ninety nanograms, or 15%, of the cDNA was collected in this fraction (Figure 2-5). Following generation of 180 ng of ds cDNA, 66 ng of size-selected (>500 bp) cDNA was obtained. Twenty five nanograms of this subtracted cDNA was ligated to 500 ng of gt11 arms, and after packaging and titering, it was estimated that there were 211,000 p.f.u. in this subtracted library.

To characterize the libraries, randomly selected clones from both the induced and subtracted libraries were selected and purified. DNA from these clones was purified, and cleaved with the restriction endonuclease EcoR I to release the cDNA insert from the gt11 arms. These DNA samples were size-fractionated by electrophoresis on a 1% agarose

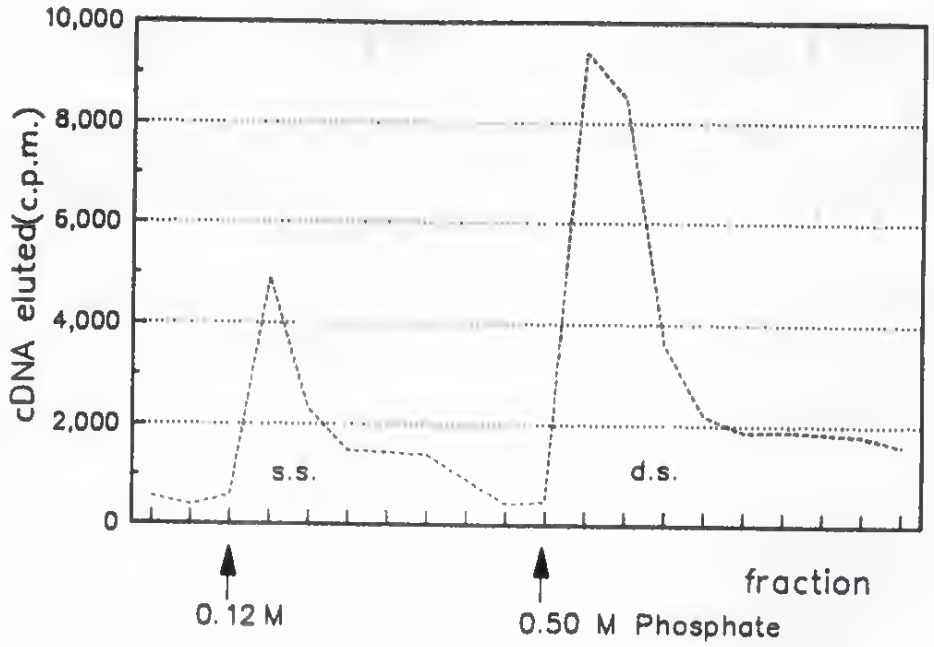

Figure 2-5. Hydroxyapatite Chromatography Profile. Separation of single-stranded and double-stranded fractions of cDNA and cDNA/mRNA hybrids used to prepare cDNA enriched for sequences corresponding to induced mRNAs. After hybridization to a R_0t value of 1500, the hybridization mix was diluted to 0.01 M phosphate and passed over the column which was preequilibrated to 0.01 M phosphate, 65°C. The single-stranded fraction was eluted with 0.12 M phosphate. cDNA eluted from the column was quantitated by liquid scintillation counting of incorporated radioactivity.

TBE gel, and stained with ethidium bromide to visualize the sizes of the inserts (Figure 2-6). The induced library had an average insert size of 1100 ± 400 bp (n=8), while the inserts from the subtracted library were somewhat shorter, the average being 900 ± 300 bp (n=9) (Table 2-1). These numbers compare well to commercial suppliers of cDNA phage libraries, such as Clontech.

The induced library was produced first, and was the first to be screened by the plus/minus method. As the limit for detection of clones using reverse transcribed cDNA probes is about 0.1% abundance of the total mRNA (Dworkin and Dawid, 1980a), it was decided to screen 10,000 p.f.u., which is nearly 10-fold excess to the total number of p.f.u. needed to be screened if every induced clone above that detection level could be identified. That is, theoretically, if you could only detect induced clones at the 0.1% abundance level, then on the average, screening 1000 clones should present most of the clones of 0.1% abundance or greater, and by screening ten times that many, you are over 99% sure that every clone of abundance 0.1% or higher will have been screened.

From the initial screen of the 10,000 plaques, 81 were identified as potentially exhibiting an induced hybridization signal, and those plaques were picked for purification and verification. After the first round in

Table 2-1. Properties and Characteristics of Induced and Subtracted cDNA libraries.

	induced library	subtracted library
mRNA template	10 μg	10 μg
first-strand cDNA	0.9 μg	0.6 μg
subtracted cDNA for enrichment of induced clones	no	yes
double-stranded cDNA insert	600 ng	66 ng
independent p.f.u.	1.3×10^6	2.1×10^5
average insert size	1100 ± 400 (n = 8)	900 ± 300 (n = 9)

Comparison of the two lambda gt11 libraries made for the purpose of differential screening. In both cases, the source of the mRNA was amino acid-starved Fao hepatoma cells.

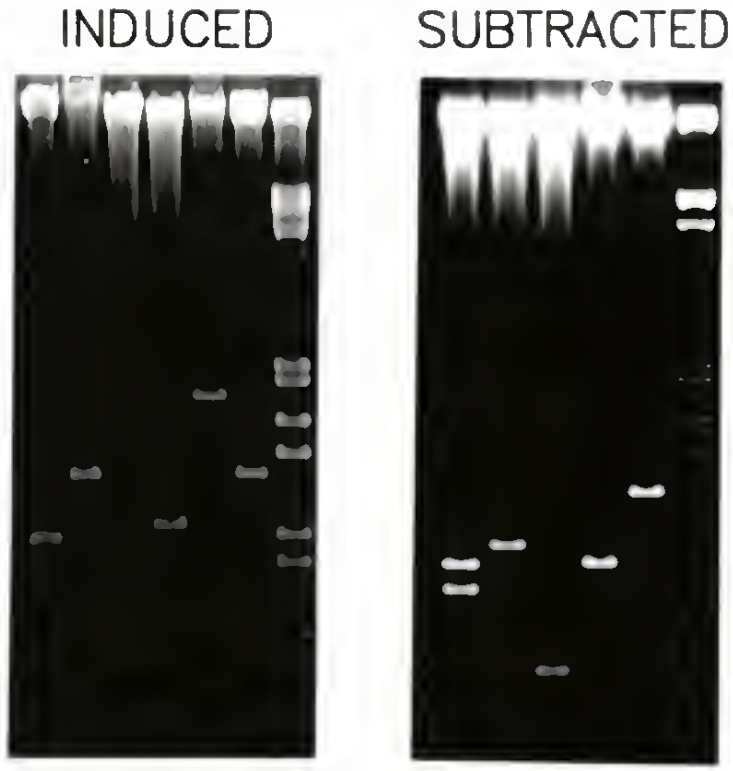

Figure 2-6. Insert characterization of cDNA libraries. Agarose gel analysis from the induced and subtracted Fao lambda gt11 libraries. DNA samples were prepared as described and digested with EcoR I to release the cDNA insert from the cloning site. Induced library, lanes 1-6: six random clones showing insert sizes. lane 7: DNA molecular size markers. Subtracted library, lanes 1-5: five random clones. lane 6: molecular size markers are wild type lambda DNA cleaved with Hind III and EcoR I.

which plaques were plated at 2,000 to 3,000 per 150 mm plate, subsequent rounds used 100 mm plates for the 80 potential positives, and only about 100 p.f.u. were plated per plate to insure clear separation of plaques, and thus aid the purification of phage clones. Of the 80 clones identified and purified, only two clones, numbers 1 and 58 (or I-1 and I-58) maintained the differential hybridization pattern through the three rounds of purification (Figure 2-7). cDNA inserts from these two clones were purified, and used to prepare [^{32}-P]-labelled probes to test amino acid-fed and amino acid-starved mRNAs for abundance of the corresponding mRNA using Northern Analysis (see Chapter 3).

The subtracted library was also screened by the method of differential hybridization using first-strand cDNA probes derived from amino acid-fed and amino acid starved cells. It was decided to screen this library at a even higher level of redundancy than the induced library, 50,000 p.f.u. being chosen as an appropriate number to screen. After obtaining the autoradiographs of the filters from the first round of screening, 22 spots on the films were identified as differentially hybridizing. The corresponding plaque or area from the agar plate was found and picked for purification and further screening. After three rounds of screening for verification and purification, there were four

clones exhibiting a differential signal through all rounds of screening. They were labelled S-1, S-3, S-5, and S-15. Ultimately, only clone S-5 from this screening proved to be an induced clone (see Chapter 3).

Discussion

Two libraries were made, one from total mRNA, and a second from subtracted first-strand cDNA. Both were derived from amino acid-starved Fao hepatoma cells. The libraries contained acceptable numbers of independent p.f.u., that is, they should contain cDNA clones representative of mRNAs with abundances down to at least 0.001%. A characterization of the average insert size from the two libraries showed that the inserts were of reasonable length, representing at least a considerable fraction of a typical mRNA, from which the full length could be identified using further library screening and/or primer extension-APCR cloning.

First-strand cDNA probes were made from a mix of mRNA prepared from different batches of cells. This was done in an attempt to average out high or low inductions of the mRNAs from each sample. Induction of System A transport in the Fao cells used for the mRNA preparations ranged from about 3-fold up to 17-fold, although the latter value may be an overestimation of the induction due to an unusually low sodium-independent transport determination.

Figure 2-7 Differential Hybridization. Top: Autoradiograms illustrating a partially purified amino acid starvation-induced cDNA clone. After picking potential induced clones from the first round of screening, the phage suspensions from each pick were plated at a density of 50 to 100 plaques per 10 cm dish, duplicate filter lifts made, and differentially hybridized. After washing filters, an autoradiogram of the filters was made. Autoradiogram shows enrichment of an induced clone present at about 10% purity. Arrows indicate location of induced signals. Clone illustrated above is I-1 from the induced cDNA library. Bottom: After 3 rounds of purification, the potentially induced cDNA clone I-1 was purified. Plaques picked from the second round of screening were suspended in SM buffer and replated as in previous round. Bottom photograph is of autoradiogram of purified induced clone exhibiting a differential signal despite presence of equal amounts of cDNA probe in both conditions. As only a single plaque was picked from the previous round, and every plaque on the plate shows the differential signal, purification was assumed, and confirmed by one additional round of screening (not shown).


A
AA−
AA+


B
AA−
AA+

The fact that only two clones were identified from a screening of 10,000 p.f.u. from the induced library and four clones from a screening of 50,000 clones from the subtracted library suggests that there is not a large number of easily detectable induced clones from the condition of starvation at three hours. For example, it would be expected that if there was just one mRNA species that was induced, and its abundance was at or above the 0.1% level in the induced state, then its corresponding cDNA clone should be present at about one in every 1,000 clones screened. Therefore, in the 10,000 plaques screened in the induced library one would expect the clone to appear about 10 times, and from the subtracted library screening of 50,000 plaques, an induced clone would appear about 50 times. Given that only 6 clones were identified from the two libraries, one of the following may be true. First, there may be no mRNA that is induced by starvation for three hours for which the abundance is above the 0.1% level. Second, it is possible that there are induced clones for which the abundance is above the 0.1% level, but the degree of induction (1- to 2-fold) may be relatively weak and difficult to detect by this method. There may, of course, be numerous mRNAs from the low

abundance class (< 0.1%) that are induced to a great degree that remain undetected.

The reults from this differential screening are consistent with the results of other work, also from our own lab. We have attempted to detect amino acid starvation-induced protein synthesis by combining pulse-labelling techniques with two-dimensional polyacrylamide gel elctrophoresis. Only 3 liver membrane proteins have been detected for which synthetic rates appear to be increased by 2- to 3-fold following amino acid deprivation of rat hepatocytes (Chiles et al., 1987). Together these two approaches, both designed to identify amino acid-regulated genes, argue that gene control with respect to starvation of mammalian cells is considerably different than that in bacterial or yeast in which expression of a large number of proteins is enhanced by several-fold or more.

Clearly, the fact that there is no mRNA yet identified for which the cellular content is raised by amino acid starvation leaves us without a positive internal control to verify that the mRNA preparation truly represents an "induced" condition. Our assumption in doing this work has been that the elevation of System A-mediated transport activity is an indication that the cells are induced by starvation.

Chapter 3

MONITORING OF mRNA LEVELS DURING AMINO ACID DEPRIVATION OF CELLS

Introduction

The goal of any differential hybridization is to obtain cDNA clones corresponding to mRNAs induced by a particular stimulus or condition. Our goal, of course, was to obtain clones that would allow us to learn more about the adaptive regulation response seen in cells starved for amino acids. It was hoped that the System A transporter cDNA might be one of the clones identified in the screening, although it is generally accepted that amino acid transporters are relatively rare proteins, which probably corresponds to a rare mRNA. This, if true, would make the identification of the System A cDNA more difficult. In any case, identification of induced clones allows us to characterize the mRNA induction: the amount, the time course, and the mechanism. As so few mammalian mRNAs have been shown to be regulated by substrate, the investigation of any induced clone allows us to determine if the regulation is primarily transcriptional, as seen in the lactose operon of *E. coli* (Pardee *et al.*, 1959) or if the control is mediated by transcriptional and post transcriptional components, as

elucidated in the gcn response in Saccharomyces cerevisiae (Hinnebusch, 1988). These systems are able to be regulated by the presence of a single sugar or amino acid. In mammalian systems, Poso et al., (1982) have demonstrated direct repression of protein catabolism in hepatocytes by individual amino acids. Although the regulation appears to be a direct effect of the amino acids, it is probably due to metabolic control and not a genetic mechanism. Our hope in obtaining any cDNA clone that corresponds to a regulated mRNA is that the mechanism, when fully understood, will provide us with a better appreciation of how mammalian cells respond to changes in nutrient levels.

The differential screening procedure described in Chapter 2 was performed with mRNA isolated from hepatoma cells incubated in the presence and absence of amino acids for 3 hours. We investigated the changes in abundance of our putative starvation-induced mRNA at various times after amino acid removal, both shorter and longer periods than the 3 hours used for differential screening. Through the use of cloned cDNAs available from our own and other laboratories, we also were able to look at the changes in abundance of several mRNA species that are typically used as controls, that is, they are often seen to be unchanged during experiments that change the abundance of more highly regulated mRNAs. Monitoring our own clone as well as the

control mRNAs was done using Northern analysis. Northern analysis is the name playfully given to electrophoretic separation of RNA followed by blotting and hybridization (Thomas, 1980), a variation of the method invented by Southern (1975) for the analysis of DNA fragments. Northern analysis allows one to identify the size and steady-state levels of many RNA samples at any specific time. This makes Northern analysis the method of choice to analyze content of a specific RNA in cells at different times, under different conditions, or from different tissues or cell types.

After finding an mRNA that was induced by amino acid starvation, our next goal was to investigate the mRNA induction in greater detail. Our current model for System A transporter regulation proposes that the increase in transport seen during adaptive regulation is due to increased transcription of the System A gene (Kilberg, 1986). We performed the plus-minus screening to find an mRNA which was induced primarily by increased gene transcription. However, it is known that the cellular concentration of many mRNAs is increased due to stabilization against degradation (Theil, 1990). Tests utilizing the inhibitor of translation, cycloheximide (Pestka, 1971), and the inhibitor of transcription, actinomycin D (Goldberg and Freidman, 1971), are necessary along with assays measuring active transcription (Groudine

et al., 1981) to determine accurately the mechanism of mRNA induction. These experiments are presented in this chapter.

Materials and Methods

Plasmid DNA purification

To purify small amounts of plasmid DNA the "mini-prep" method of Ish-Horowitz and Burke (1981) as described in Davis et al., (1986) was used. Briefly, cells were pelleted for 10-30 minutes at 1500g, then resuspended in a solution of 50 mM glucose, 25 mM Tris, pH 8.0, and 10 mM EDTA. Lysozyme was added to a final concentration of 2 mg/ml, and the mixture was incubated at room temperature for 5 minutes. A solution of 0.2 M NaOH and 1% SDS was added, the cells were then incubated on ice for 5 minutes. A solution of ice-cold 5 M potassium acetate, pH 4.8, was added and mixed, and then the mixture was centrifuged at 13,000g at 4°C for 10 minutes. The supernatant fraction, which contained the plasmid, was filtered through gauze, and treated with 1 µg/ml RNase A for one hour at 37°C. The samples were extracted with phenol-chloroform and the plasmid DNA precipitated with ethanol or isopropanol.

The "Triton-Lysozyme" method of plasmid purification from Davis et al., (1986) was used for large-scale preparation of plasmid DNA from one liter cultures of plasmid-containing bacteria. After pelleting cells as in the mini-prep method, a solution of 10% (w/v) sucrose, 50 mM

Tris, pH 8.0 was used to resuspend the cells. Lysozyme was added to the suspension to 3 mg/ml; this was then incubated on ice for 10 minutes. A solution of 0.5 M EDTA was added and mixed, followed by another incubation on ice for 5 minutes. At this point, Triton X-100 was added to 0.3% (w/v) and mixed, and the mixture was incubated at 37°C for at least 2 minutes or until bacterial lysis became apparent. The lysed cells were centrifuged in a Beckman Ti60 rotor at 200,000g (50,000 rpm) for 30 minutes to pellet lysed bacteria. The supernatant, containing the plasmid DNA, was RNase A treated as described above, then extracted with phenol-chloroform and ethanol precipitated.

Use of DNA restriction enzymes

DNA restriction enzymes were used as recommended by the supplier at concentrations of at least 1 unit of enzyme per microgram of DNA to be digested. In the case of lambda phage DNA or very large volumes of plasmid DNA, digestion was typically carried out overnight, with up to 5 units of enzyme per microgram of DNA.

DNA agarose gels

DNA was size-fractionated on 1% agarose gels using a 1X TBE buffer, essentially as in Maniatis et al., (1982). DNA samples were added to loading buffer containing 10% glycerol with bromophenol blue and xylene cyanol, each at 0.05% (w/v). After electrophoretic separation, DNA was stained

for 30 minutes in a 1 μg/ml solution of ethidium bromide to allow visualization of the DNA under ultraviolet light.

Electroelution of DNA fragments

The procedure from Davis et al., (1986) was used to purify DNA fragments from agarose gels. Briefly, fragments were cut from gels and placed in dialysis tubing with 1 to 3 ml of 0.2X TBE buffer depending on the size of the agarose block cut from the gel. The tubing was clamped and placed in an electrophoresis box with enough 0.2X TBE buffer to completely cover the tubing. The voltage applied was 100 to 300 volts, depending on the size of the gel box used, and electroelution was carried out for 1 to 3 hours, depending on the size of the fragment in the agarose. Upon completion of electroelution, the polarity on the gel box was reversed and voltage was reapplied for 2 minutes to remove DNA fragments from the inner surface of the dialysis tubing. The fluid was removed from the dialysis bag by pipetting, and the volume decreased to 0.4 ml using sec-butyl alcohol extractions to remove water from the DNA solution. The volume was phenol-chloroform extracted and precipitated overnight with an equal volume of isopropanol. The DNA was pelleted at 13,000g for 30 minutes, and the pellet rinsed with 80% (v/v) ethanol, then air-dried. The pellet was dissolved in 10 to 100 μl of TE, and 2 μl of this solution were run on a mini-gel along with molecular size markers of

known concentration to confirm purification of the insert and to estimate the concentration of the insert.

RNA gel electrophoresis

RNA was size-fractionated on horizontal agarose gels consisting of 1% (w/v) agarose, 0.04 M morpholinopropanesulfonic acid (MOPS), pH 7.0, 0.01 M sodium acetate, 2.2 M formaldehyde and 1 mM EDTA. RNA samples (typically 1 to 20 μg) were mixed with a loading buffer containing the above buffer plus 50% (v/v) formamide, 0.5% (w/v) bromophenol blue and 10% (v/v) glycerol. After heating the samples for 15 minutes at 65°C, 1 μl of a 1 mg/ml ethidium bromide solution was added to each sample, mixed, and loaded on the gel. Typically, gels were run overnight at 40 volts with buffer recirculation, although shorter runs at higher voltages gave similar resolution.

RNA blotting

We used "Gene Screen" nylon paper for blotting and used the following modification of a blotting protocol supplied by the manufacturer (DuPont/NEN). Following electrophoresis, gels were incubated in distilled water for a total of 10 minutes with several changes of the water, then incubated for 15 to 30 minutes in 50 mM NaOH, followed by a 30 minute incubation in 100 mM Tris, pH 7. The gel was then laid upside down on a wick made from 3 mm Whatman paper, and the nylon membrane, which was previously

incubated in 10X SSPE for 10 minutes, was applied to the surface of the gel. The RNA was then transferred to the nylon membrane by capillary action (Davis et al., 1986) using 10X SSPE as the transfer buffer and paper towels above the nylon membrane to drive capillary flow. The next day, the transfer was confirmed by inspection of the blot and gel on an ultraviolet light box, then the RNA was covalently cross-linked to the membrane by exposing the blot to ultraviolet light for 3.5 minutes at a distance of 40 cm. Membranes were stored damp, wrapped in plastic wrap at 4°C until needed.

Northern hybridization

Hybridizations used nylon membranes prepared as described above and [^{32}P]-labelled cDNA fragments to detect relative mRNA abundance by autoradiographic analysis. Radiolabelled probes were prepared as described below, and added to the hybridization solution to achieve a concentration of at least 10^6 cpm of probe per ml of hybridization solution. Hybridization solution was 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, 1% (w/v) Fraction V bovine serum albumin (BSA) and 7% (w/v) SDS (Church and Gilbert, 1984).

Prior to hybridization, membranes were pretreated as follows: The membrane was washed in 20 mM Tris, pH 7.0-7.5 at room temperature. The Tris was heated to 95°C, then

poured on the membrane which was incubated on a rocking platform at room temperature for 10 minutes. Following this wash, the blot was placed in 0.1X SSPE, 0.1% SDS for one hour at 65°C. This pretreatment enhances the hybridization signal and allows for better detection by autoradiography (Ecker and Davis, 1987). After hybridization overnight at 65°C, membranes were washed 3 times at room temperature in hybridization wash solution A containing 0.5% BSA, 40 mM sodium phosphate, pH 7.2, 5% SDS, and 1 mM EDTA. Following these washes, blots were washed for one hour at 65°C in the hybridization wash solution B: 40 mM sodium phosphate, pH 7.2, 1% SDS, and 1 mM EDTA. Membranes were then blotted dry and subjected to autoradiography.

Blots previously hybridized were stripped for rehybridization by boiling in a solution of 0.1X SSC, 1% SDS for one hour. We found it useful to verify the stripping by placing the stripped blot on film for one to two days to confirm that no radioactivity was left on the filter.

Control probes

The ß-actin, glyceraldehyde phosphate dehydrogenase, histone H4 and Cu-Zn superoxide dismutase cDNA inserts were obtained from the laboratory of Dr. H. S. Nick. Recombinant plasmids containing coding sequences for these genes were grown using E.coli host strain DH5α in liquid culture with the appropriate antibiotic specific to each plasmid.

Plasmids and cDNA inserts were purified as described above.

Preparation of [^{32}-P]-labelled DNA probes

We used the "random-primer extension" method of Feinberg and Vogelstein (1983) using a manufacturer's reagent kit (BRL). We found that by using this kit, we consistently obtained incorporation of radiolabel in excess of 50% when labelling 50 to 100 ng of double-stranded DNA insert. After boiling to denature the DNA, the insert was incubated at room temperature for at least 2 hours in the following solution: 20 μM of dATP, dGTP, and dTTP in 0.2 M HEPES, 50 mM Tris, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.4 mg/ml BSA, 5 OD_{260}units/ml oligodeoxynucleotide primers (hexamer fraction), pH 6.8. Fifty microcuries of [α-^{32}-P]dCTP, 3000 Ci/mmol, 10 μCi/μl and 3 units of Klenow fragment were added in a final volume of 50 μl.

Incorporation of radiolabelled dCTP into DNA probe was assayed as follows: two microliters of the probe mixture were removed and diluted with 498 μl of TE buffer, then 10 μl of this dilution were pipetted into a 15 ml disposable plastic test tube. Ten milliliters of ice-cold 10% (w/v) TCA were added to the tube, mixed by inversion, and then the mixture was filtered through a glass-fiber filter disc (Whatman Type GFC). The tube was washed with 10 ml of ethanol; this volume was filtered through the disc followed by 10 ml of acetone. A second filter was directly spotted

with an additional 10 µl of the diluted probe mix. The two filters were put into vials for liquid scintillation counting. Total incorporation and percent incorporation were determined by comparing the TCA-precipitated counts to the total counts.

We found it unneccessary to purify the probe any further for Northern hybridizations. After analysis of incorporation, probes were boiled for 5 minutes to denature the double-stranded DNA prior to adding the probe to the hybridization solution. After hybridization, the probe mixture was removed and saved frozen at -20°C. The frozen probe mix could be used up to two weeks later for a subsequent hybridization to mRNAs of reasonable abundance.

Subcloning of cDNA inserts into plasmids

We essentially followed the method of Hanahan (1983). A 100 ng aliquot of EcoR I-digested plasmid DNA was added to a 3-fold molar excess of cDNA insert. The cDNA was ligated to the plasmid for at least 2 hours at room temperature, or overnight at 15°C for blunt-end ligations, in a buffer containing 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, and 5% (w/v) polyethylene glycol-8000, which optimizes ligation for subsequent transformation (King and Blakesley, 1986).

Transformation of bacteria

Ligation mixtures containing recombinant plasmids were used to transform E. coli strain DH5α (Hanahan, 1983) which was obtained as competent cells from Bethesda Research Laboratories, Bethesda, Maryland. The protocol supplied from the manufacturer was followed: incubation of 50 μl of cells mixed with 2 μl of ligation mixture on ice for 45 minutes, followed by a 30 second incubation at 37°C, then 2 more minutes on ice. The mixture was added to 1 ml of LB medium and incubated at 37°C for one hour. The cells in this volume were pelleted with a one minute centrifugation at 13,000g and the cell pellet was gently resuspended in 100 μl of LB. Cells were then spread on a plate of LB containing 1.5% (w/v) agar, with 50 μg/ml ampicillin, 0.025% (w/v) IPTG, and 0.025% (w/v) X-gal. The plates were incubated overnight at 37°C. Because DH5α is ampicillin-sensitive, only bacteria containing the plasmid will grow; blue colonies indicate plasmid without an insert, white colonies indicate a plasmid with insert.

Amino acid starvation of Fao and other cells in culture

The starvation-induced response of the ASI-1 mRNA was optimized as follows: Cells were trypsinized from a confluent 150 cm^2 culture flask and suspended in 50 ml of MEM supplemented with 6% (v/v) fetal bovine serum (fbs). An aliquot of the suspension was used to determine the number

of cells/ml using a "SPot lite" counting chamber (Hausser Scientific, Blue Bell, Pennsylvania). After the cell density was determined, 4 x 10^6 cells were plated into 10 cm round plastic culture dishes; the total volume of MEM plus FBS in each dish was brought up to 12 ml by adding extra medium. The cells were cultured for 48 hours before changing the medium to begin an experiment. Whenever new medium was added to cells, the cells would be rinsed two times in the new medium, aspirating off each rinse, followed by the addition of the medium for the experiment. Initially the amino acid-starved and -fed conditions were achieved by using NaKRB with or without the following six amino acids: alanine, glycine, serine, threonine, proline, and asparagine, all at 3 mM. Later experiments used sodium-containing Krebs-Ringer bicarbonate buffer (NaKRB) for the amino acid-starved condition and MEM without FBS for the amino acid-fed condition. Experiments using vitamins were done by adding 100X liquid vitamin stock (Sigma) to the culture media. Inhibitors actinomycin D (25 μM), cycloheximide (100 μM), L-azetidine-2-carboxylic acid (10 mM), and histidinol (10 mM), were added to 50 ml of culture media 24 hours prior to their use to insure complete dissolution into the media. Starvation and other time course experiments were performed for time periods up to 12 hours. RNA was prepared from cells in culture by addition

of the denaturing guanidinium solution ("solution D") directly onto cells adhering to the culture plate, and then following the procedure described in Chapter 2.

Quantitation of autoradiograms

Relative abundances of mRNAs as determined by Northern analyses were quantitated using the LKB Ultroscan XL laser densitometer. Bands or spots on developed X-ray film were scanned using the 2-dimensional scanning mode of the densitometer, taking care to use exposures that produced bands that were neither underexposed nor overexposed, so that the exposures were in the linear range of detection. Resultant intensities were given as absorbance units/mm^2, and values for various bands from an experiment were expressed as a ratio to control values and expressed as relative units.

Nuclear run-off transcription assay

To analyze active gene transcription, we used the "nuclear runoff" method (Marzluff and Huang, 1985) as described by Groudine et al, (1981) and modified by Ausubel et al, (1987) with our own further modification involving use of the "single-step" purification of RNA (Chomczynski and Sacchi, 1987) to isolate radiolabelled mRNA from nuclei.

Fao cells were prepared as described previously, plating 9 x 10^6 cells per 150 mm round culture dish 48 hours prior to the start of an experiment. Upon starting an

experiment, MEM containing 6% fbs was replaced with either MEM (no serum) for the amino acid-containing condition, or NaKRB (no serum) for the amino acid-free condition. Dishes were rinsed twice with one volume of the new medium before adding a third volume to start the experiment. Two dishes per condition were prepared to obtain adequate numbers of cells for RNA labelling. At appropriate times, dishes were removed from the incubator, culture medium drained and replaced with ice-cold PBS, and the dish placed on a wet ice slurry. Cells were scraped from the dish with a plastic "Bondo" spreader. The PBS/cell suspension was collected into a sterile 15 ml plastic tube and centrifuged for 5 minutes at 1000g to pellet the cells. To the pellet, 4 ml of Nonidet P-40 (NP-40) lysis buffer (NP-40 lysis buffer is 10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40) were added dropwise while the tube was vortexing at half-maximal speed. The tube was then vortexed for an additional 10 seconds. After sitting on ice for 5 minutes, the mixture was centrifuged for 5 minutes at 1000g to pellet the nuclei. The pelleted nuclei were resuspended in NP-40 buffer and centrifuged again as described above. The pellet from this centrifugation was saved and resuspended in 200 μl of glycerol storage buffer (glycerol storage buffer is 50 mM Tris, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA). Two hundred microliters of 2X reaction buffer (2X reaction

buffer is 10 mM Tris, pH 8.0, 5 mM $MgCl_2$, 0.3 M KCl, 5 mM DTT, and 1 mM each of ATP, CTP, and GTP) were added to this volume along with 10 µl of [α-^{32}P]UTP (800 Ci/mmol, 40 mCi/ml). After mixing, the nuclear suspension was incubated in a shaking water bath at 30°C for 30 minutes to allow nuclear transcripts to elongate, incorporating the radiolabelled UTP. After this incubation, RNA was purified from the suspension using 4 ml of Solution D using the method of Chomczynski and Sacchi (1987), described in detail in Chapter 2. The resultant RNA pellet from this procedure was dissolved in 100 µl of DEPC-treated water, and 5 µl were removed and counted by liquid scintillation spectrometry to determine the radioactivity incorporated into the transcripts. Equal amounts of radioactive counts were used for each hybridization, typically 5-10 x 10^6 cpm. An appropriate volume of the radiolabelled-RNA was added to 1.5 ml of hybridization buffer (as described above) and mixed. Then a nylon filter dot- or slot-blot containing 10 µg of each plasmid (either the ASI cDNA insert, the ß-actin cDNA insert or the pUC 19 plasmid serving as a control) was placed in a microfuge tube containing the hybridization solution. The tube was then incubated for 48-72 hours at 65°C to allow hybridization of radiolabelled-mRNAs to their corresponding cDNAs on the filter. After hybridization, the filters were washed three times for 15 minutes each in 50 ml

of wash solution B (described above) at 65°C. Washed filters were subjected to autoradiography. In a cassette containing Cronex "Lightning-Plus" intensifying screens, typical exposure times of the Kodak XAR film were 1 to 7 days. Relative rates of transcription were determined using the LKB Ultroscan densitometer to quantitate the amount of hybridization between different conditions.

Results

Confirmation of a starvation-induced mRNA

Although 3 bacteriophage cDNA-containing clones had survived three rounds of purification exhibiting the differential hybridization pattern that would be expected of a starvation-induced clone, only one of those, clone S-5, showed the differential, induced pattern as determined by Northern analysis. Phage DNA was prepared from this clone and 100 μg of this DNA were digested overnight using 150 units of the restriction enzyme EcoR I. The digest was phenol-chloroform extracted, pelleted and taken up in TE buffer. The digested DNA was size-fractionated on a 1% agarose, TBE gel at 30 volts overnight, and the digested phage arms and an approximately 450 bp insert were detectable on the gel. The insert was cut from the gel and electroeluted as described. The insert was subsequently subcloned into the plasmid vector pGEM-3Z (Figure 3-1) as described in the methods section of this chapter.

To confirm the induction of this clone, a [^{32}P]-labelled probe was made from the purified insert and hybridized to a Northern blot containing two lanes of RNA, one with RNA prepared from Fao cells starved for amino acids for three hours, the second with RNA from cells prepared identically, except that amino acids were always present. To confirm an equal loading of RNA onto the two lanes of the blot, a similarly radiolabelled-probe for the ß-actin mRNA was prepared and added to the hybridization. When positive results were obtained, a second hybridization was conducted with another blot containing the same two conditions, but from a second preparation of cellular RNA. From the two experiments, the mRNA for S-5 was induced an average of 1.5-fold by starvation, whereas the actin mRNA was quantitated to be reduced to 0.65-fold of its fed level. The combination of these two factors yielded a 2.3-fold level of induction if expressed as a ratio of S-5 to actin. A third experiment was performed to verify these results, and again the results confirmed the first two trials. The average of the three experiments was a 2.1-fold induction of S-5 as compared to expression of ß-actin (Figure 3-2). At this point, confident that an induced clone had been identified, we renamed clone S-5 as ASI, for amino acid-starvation induced clone.

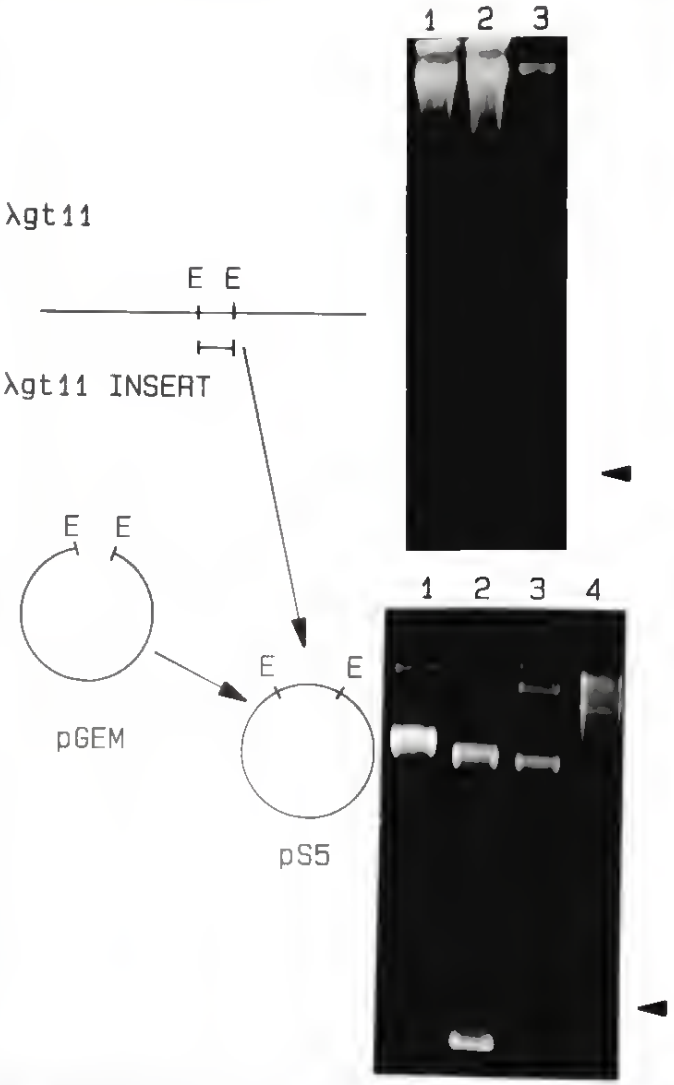

Figure 3-1. Subcloning of the Fao hepatoma (S-5) ASI cDNA insert. DNA purified from lambda clone S-5 was digested with restriction endonuclease EcoR I and size-fractionated on a 1% agarose gel. The 425 bp fragment was electroeluted from the gel and ligated into the EcoR I site of plasmid pGEM-3Z. Top, lambda DNA: lane 1, undigested wild type DNA; lane 2, digested S-5 DNA; lane 3, molecular size standards. Bottom, recombinant pGEM plasmid: lane 1, linearized plasmid; lane 2, EcoR I digested plasmid; lane 3, undigested plasmid; lane 4, molecular size standards. Arrows mark location of 564 bp DNA standard from lambda EcoR I/Hind III size markers.

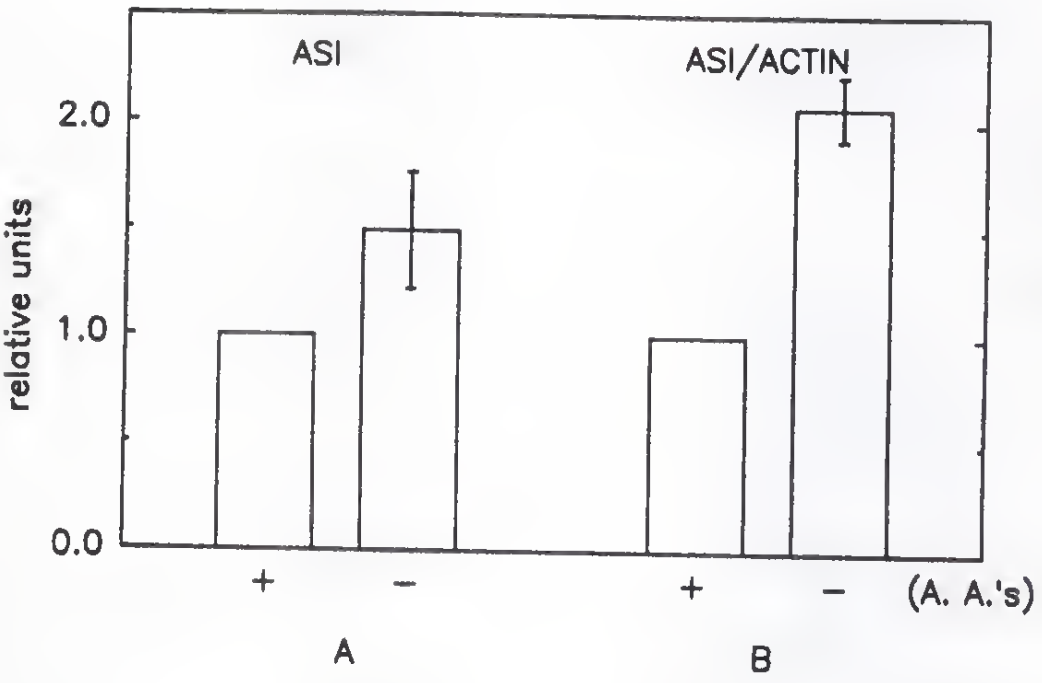

Figure 3-2. Northern analysis of ASI mRNA induction. ASI mRNA abundance after 3 hours of hepatoma culture in amino acid-free (NaKRB) or -supplemented (NaKRB + 6 amino acids) media. A: abundance of ASI mRNA after 3 hours with or without amino acids. B: Ratio of ASI mRNA abundance compared to ß-actin abundance, with or without amino acids. Quantitation of mRNA abundance was determined using laser scanning densitometry of three independent Northern analyses.

As the induction of ASI was small, a series of experiments were undertaken to determine if under other conditions, the induction of the ASI mRNA would be greater than that observed at three hours. The first experiment performed was to extend the period of amino acid-starvation beyond three hours, and this again provided us with positive results. When the starvation period was extended to longer times, greater inductions were found. Numerous experiments showed us that the magnitude of this induction was highly variable, from 2-fold to 4- to 5-fold at its maximum. The ratio during amino acid starvation of ASI to actin never decreased below the starting ratio, and we found that the increase would occur by 9 hours of starvation. Although experiments were conducted in which cells were starved for longer than 12 hours (data not shown), it was found that the survival of Fao cells diminished significantly beyond this time point. Therefore, we chose to limit our time course experiments to 12 hours.

The variability in the induction of ASI proved to be due to variation in the initial condition of the cells when a starvation experiment was begun, with the age of the cells on the plate and their density on the plate being major factors in the cells' ability to produce the induction of

the ASI mRNA. Experiments were performed which varied the number of cells plated onto the culture dishes and measured the induction as a function of that variable, and the following protocol produced a consistent induction: cells were plated 48 hours prior to the beginning of an experiment at a density of 4×10^6 cells per 10 cm culture dish in MEM plus 6% FBS. After 48 hours of growth on the plates, the MEM plus FBS was removed and the experimental culture media were added to the plates. Dishes were rinsed twice with the new culture medium before leaving a third volume of medium on the cells to begin the experimental condition. Figure 3-3 shows what we consider to be the optimized induction of the ASI mRNA. From the initial time, the ASI mRNA abundance increases from its starting level to an amount between 2- and 3-fold higher, and if the induction is compared to the level of actin mRNA during the time course, then the relative level of the ASI mRNA was about 8-fold above its starting value. This induction of ASI was complete by 9 to 12 hours. We note that the actin mRNA decreased as a result of amino acid starvation; a typical change in actin was a drop of one-half to one-fourth of its original abundance after 12 hours of amino acid starvation.

Because we were not sure if the decrease in the actin mRNA was a specific, unique response by this gene or representative of a general phenomenon of mRNA decrease due

to starvation, we performed Northern analyses of Fao cells starved for amino acids for up to 12 hours. RNA was purified from cells at various times during the experiment. The RNA samples were then used to prepare a blot of cells starved for amino acids, fed with amino acids, and fed with amino acids and supplemented with 6% fbs. This blot was then probed with radiolabelled DNA probes that are typically used by other laboratories in Northern analysis as control probes -- that is, they are rarely changed to any great extent by many cellular conditions. In addition to actin, we used histone H4, glyceraldehyde 3-phosphate dehydrogenase (GPD), and copper-zinc superoxide dismutase (SOD). We observed that all four mRNAs decreased significantly due to amino acid starvation, and remained relatively constant when cultured in the presence of amino acids (Figure 3-5), except for histone H4, which decreased even in the presence of amino acids and did not remain constant unless fbs was added to the medium (data not shown). By 2 to 4 hours, there are slight decreases seen in mRNA abundances for all four of the control probes, and by 6 to 9 hours, significant decreases were seen. In the fed condition, the mRNA levels for actin, GPD, and SOD remained constant throughout the experiment, while the decrease in histone is seen after 4 hours and decreased in a linear fashion through 12 hours.

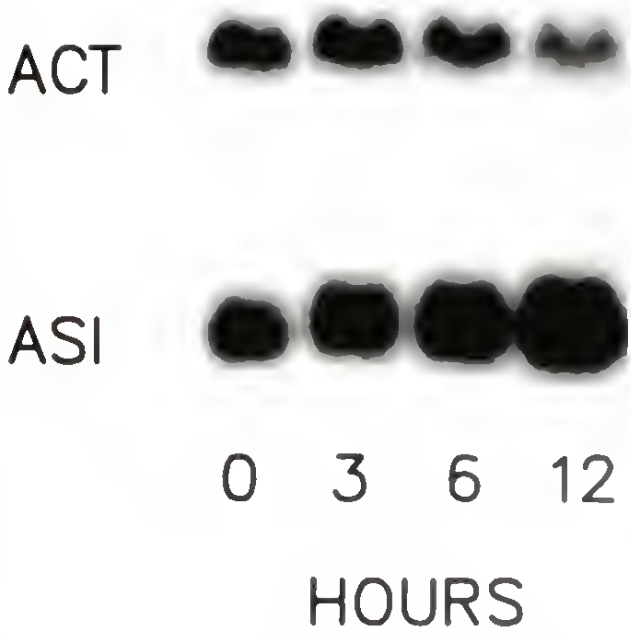

Figure 3-3. Induction of ASI mRNA by amino acid starvation of rat hepatoma cells. Fao cells were cultured in amino acid-free media (NaKRB) for 12 hours, with RNA being purified from the cells at 0, 3, 6, and 12 hours. Northern analsis shows relative abundance of ASI and ß-actin (ACT) mRNAs during amino acid starvation.

Another set of experiments was undertaken to examine the reversibility of the induction of ASI-1 by starvation. Cells were prepared as described previously, and two groups of Fao cells were maintained in either NaKRB (amino acid-starved) or MEM (amino acid-fed) for 12 hours. After 12 hours, the cells from both conditions had their culture medium withdrawn and replaced with amino acid-containing MEM. These cells were cultured for an additional 24 hours. RNA was prepared from both groups of cells at 0, 3, 6, 9, and 12 hours of the starvation period, and then after 12 and 24 hours of the "re-fed" state, which are hours 24 and 36 of the experiment from time zero. The Northern analysis is shown in Figure 3-6. The ASI-1 mRNA, after a slight decrease from zero to 3 hours, increased approximately 3-fold over the initial abundance after 9 to 12 hours of starvation. Actin during this same time period decreased well below its initial value. The actin and ASI-1 mRNA abundance was constant for the amino acid-fed cells throughout the course of the experiment. Upon refeeding of amino acids to the starved cells, two changes were noted. The first change was a decrease of the ASI-1 abundance, although the ASI mRNA abundance did not drop to its initial level. The second change was larger, an increase in the actin mRNA of over 10-fold in the first 12 hours of refeeding as compared to the abundance at the 12th hour

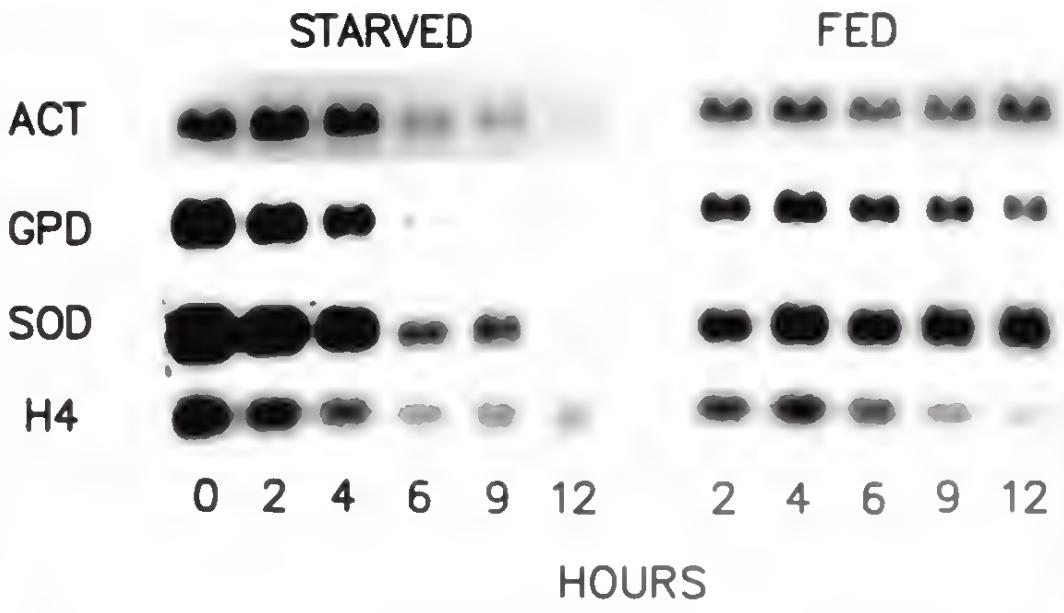

Figure 3-4. Abundance of several mRNAs during amino acid starvation of rat Fao hepatoma cells. Fao cells were cultured in amino acid-free ("starved") or -supplemented ("fed") culture medium for various time periods, and RNA was then purified from the cells. Total cellular RNA was size-fractionated, blotted, and used for hybridization with the following cDNAs: ACT: ß-actin, GPD: glyceraldehyde-3-phosphate dehydrogenase, SOD: Cu-Zn superoxide dismutase, H4: histone H4.

of starvation. The increase in actin due to refeeding produced an mRNA abundance that was approximately 3-fold above the initial abundance of the actin message.

A summary graph representing averaged values for the ASI-1 and actin mRNAs is shown in Figure 3-6. Data was analyzed by laser scanning densitometry of Northern analyses performed using mRNA prepared from amino acid-starved or -fed Fao cells. Figure 3-7 presents results of four separate Northern analyses which were performed to confirm that changing the amino acid-fed cell medium from NaKRB supplemented with six amino acids at 3 mM each to MEM, which contains all of the amino acids, did not alter the basic observation.

The only significant difference between NaKRB (plus the six added amino acids) and MEM, except for their amino acid differences, is that MEM contains vitamins essential for cell growth, whereas NaKRB does not. The experiment shown in Figure 3-7 involved the addition of a vitamin supplement to NaKRB to bring the concentration of vitamins in that medium up to the concentration in MEM, then starving cells for amino acids using NaKRB with and without the vitamin supplement. If the induction of ASI-1 occurred in both instances, then the presence of vitamins in MEM could be eliminated as the factor that repressed the induction of

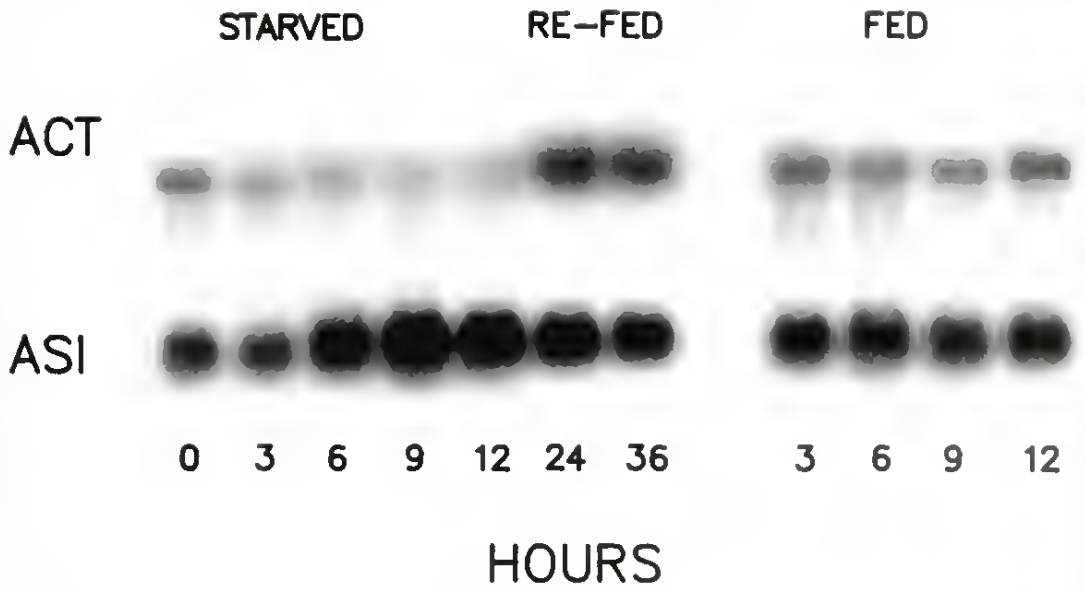

Figure 3-5. Refeeding effects on ASI expression in hepatoma cells. Fao cells were cultured in amino acid-free or -supplemented medium. RNA was purified from the cells at the indicated times for Northern analysis. After cells were incubated for 12 hours in amino acid-free medium ("starved"), the culture medium was replaced with one containing an amino acid supplement and then the cells were incubated for an additional 24 hours ("re-fed").

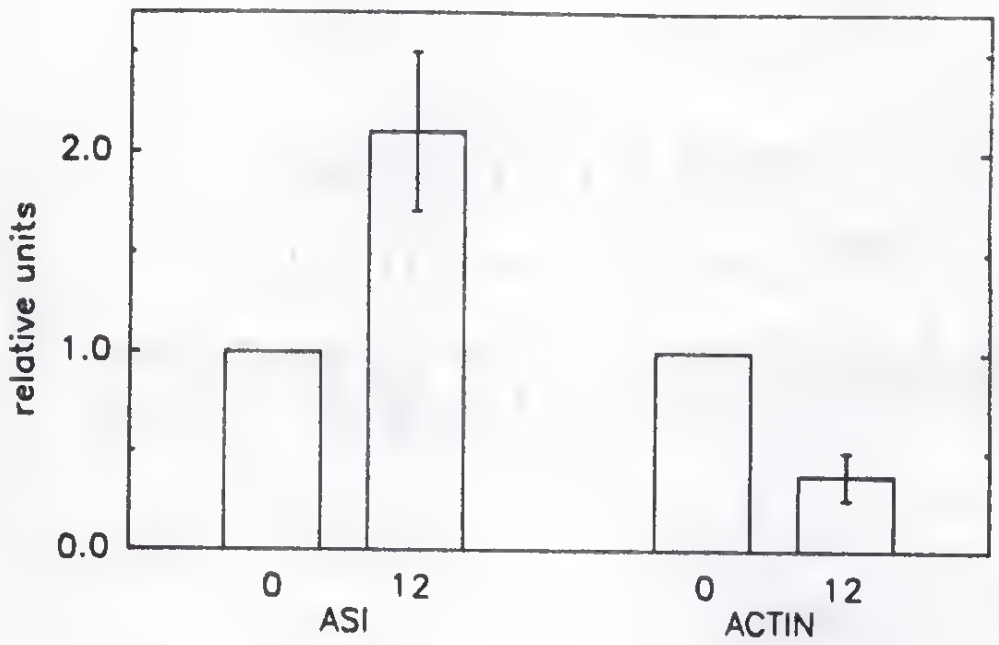

Fig. 3-6. Quantitation of actin and ASI mRNA levels after starvation of Fao hepatoma cells for 12 hours. Relative abundances of ASI and actin mRNAs after 12 hours of amino acid starvation are expressed as relative absorbance units as determined by laser scanning densitometry of autoradiographs of Northern analyses. ASI data are from 5 independent experiments, whereas the actin data are from 3 separate experiments.

ASI-1. The results presented in Figure 3-7 confirm that amino acids rather than vitamins regulate the expression of ASI-1. The induction of ASI-1, as compared to actin, was 3.2-fold (±1.7) after 12 hours of culture in NaKRB plus vitamins compared to MEM. The average induction in NaKRB without vitamins at 12 hours of starvation was 4.3-fold (±0.8). After refeeding the amino acid-starved cells, the relative abundance dropped to 1.5 ± 0.4. Although the average induction without vitamins was slightly higher, a hypothesis test between the difference of those two means using the t-distribution test (Merrington, 1941) showed that the two numbers were statistically indistinguishable ($p<0.01$). After considering the results of these experiments, all subsequent starvation experiments used NaKRB for the amino acid-free medium and MEM as the amino acid-containing medium.

Having characterized the time course of the induction of the ASI-1 mRNA, we proceeded to conduct a series of experiments to investigate the nature of the ASI mRNA induction. The inhibitor of transcription, actinomycin D, and the translation inhibitor cycloheximide were used in conjunction with Fao cells to follow the induction of ASI-1 by Northern analyses at various times after starvation. Actinomycin D, used at 25 μM, has been shown to be an effective inhibitor of mRNA synthesis (Goldberg and

Freidman, 1971), and cycloheximide, at 100 μM, an equally effective inhibitor of protein synthesis (Pestka, 1971). Using the two inhibitors at these concentrations, a series of experiments were completed to determine if the induction of the ASI-1 mRNA was dependent on either active transcription and/or translation. Northern analyses were performed, and the autoradiograph from each hybridization was scanned using laser scanning densitometry to normalize each condition and time point to the initial time point ("t=0") from that experiment. A summary of the results of this series of experiments is shown in Figure 3-8. As presented previously in bar graph form (Fig. 3-6), the induction of the ASI mRNA rose just over 2-fold by 9 hours of starvation compared to a constant amount of the mRNA in cells supplied with amino acids. Actinomycin D caused the ASI mRNA to decay to approximately one-third of its initial abundance at 9 hours in both the starved and fed cases. In contrast, the presence of cycloheximide stabilized the ASI mRNA abundance at its initial level. In both the starved and fed cases, when cycloheximide was present, the relative abundance of the ASI mRNA remained near its starting level.

The abundance of ß-actin mRNA was also regulated by presence or absence of amino acids, but in the opposite manner to the ASI mRNA. Actin mRNA levels remained constant in the presence of amino acids, but dropped to approximately

one-fourth of their initial value at 9 hours of amino acid starvation. The presence of either actinomycin D or cycloheximide caused the actin mRNA to drop in abundance to about one-half of its initial value by 9 hours of culture. There did not appear to be any significant difference between the two inhibitors with respect to changes in actin mRNA levels, in contrast to the change in abundance with or without amino acids. The latter effect was statistically significant and reproducible from experiment to experiment.

The metabolic inhibitors L-azetidine-2-carboxylic acid and histidinol are amino acid analogs of proline and histidine, respectively, that are incorporated into nascent polypeptides, preventing any further elongation of the growing polypeptide. In this respect, these compounds mimic starvation for the two amino acids, as translation is stalled when the proline or histidine analog is incorporated. We chose to use the analogs as a supplement to the fed condition, so that along with a normal level of the amino acids proline and histidine, there would be included, at 10 mM, either one of the two polypeptide chain terminators. Fao cells were incubated as before, and after 48 hours of growth in round culture dishes the media was removed and replaced with either NaKRB (amino acid-free), MEM (amino acid-fed), or MEM plus either one of the

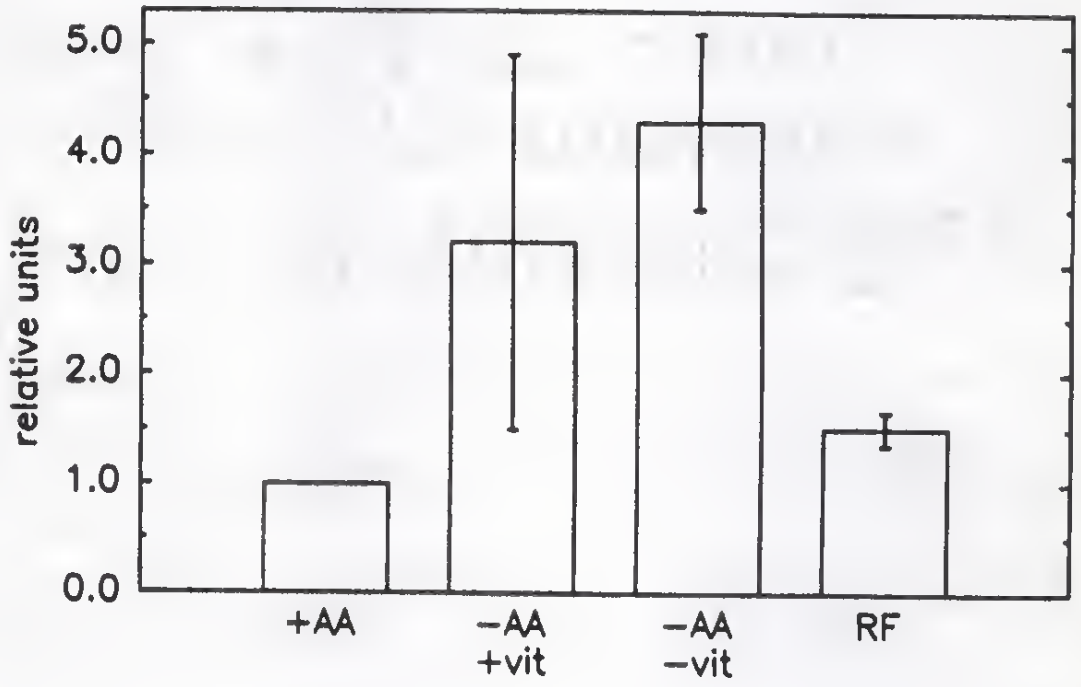

Fig. 3-7. Quantitation of ASI mRNA induction in amino acid-free media with or without vitamins. +AA: Fao cells cultured for 12 hours in amino acid-supplemented media with vitamins. -AA: Cells incubated for 12 hours in amino acid-free media with (+vit) or without (-vit) vitamins. RF: Cells were incubated for 12 hours in the absence of amino acids and then transferred to media containing amino acids and vitamins for an additional 12 hours in amino acid-supplemented media with vitamins. Following Northern analysis, the quantitation of autoradiographs was by laser scanning densitometry.

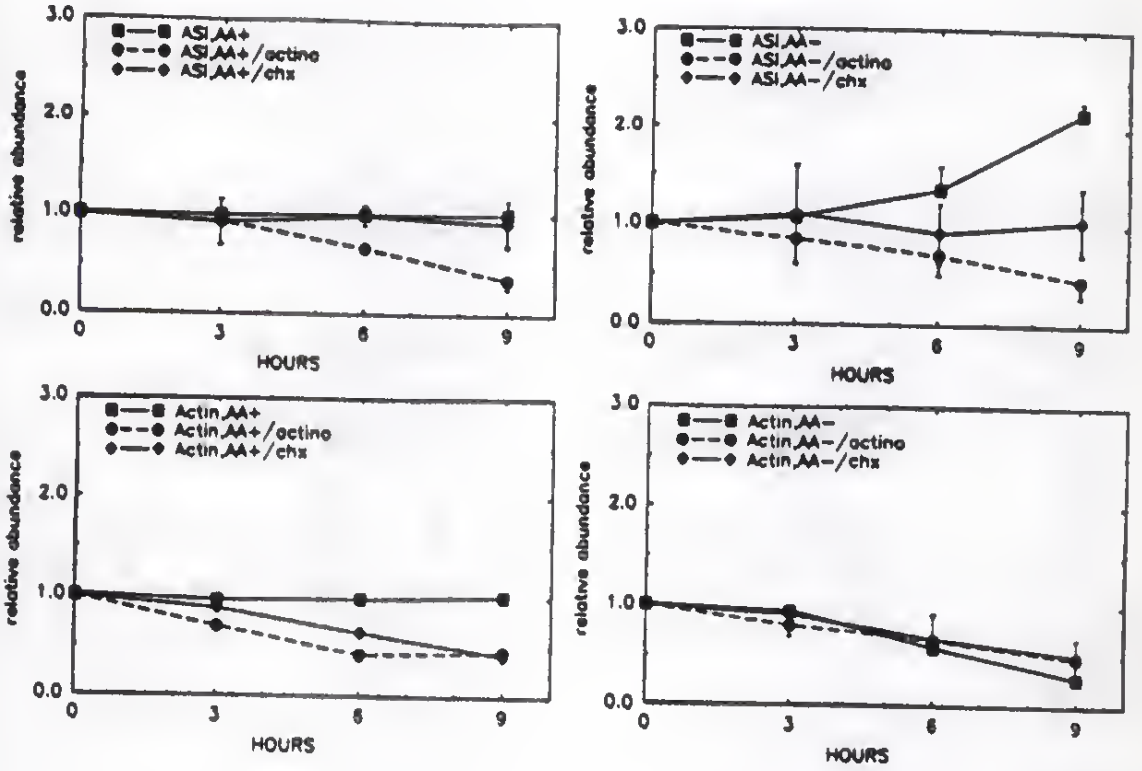

Figure 3-8. Effect of metabolic inhibitors on the induction of ASI mRNA in Fao cells. Amino acid starvation of Fao cells was performed in the presence of the transcription inhibitor actinomycin D (25 μM) or the translation inhibitor cycloheximide (100 μM). Quantitation of a series of Northern analyses was done using laser scanning densitometry. Each time point is the average of 2 or 3 determinations and standard deviations where not shown are contained within the symbol.

inhibitors at 10 mM. The cells were then cultured for 12 hours, at which time RNA was purified from the cells and used for Northern analysis. The average of two identical analyses showed that in response to amino acid deprivation the ASI mRNA was induced 2.55-fold (± 0.30) over the fed condition (Fig. 3-9). With the inclusion of L-azetidine-2-carboxylic acid to MEM, the ASI mRNA was induced 1.81-fold (± 0.04) over the fed condition. Histidinol produced a 1.65-fold (± 0.23) elevation over the level in fed cells.

Additional experiments were pursued to investigate the potential involvement of individual amino acids in the repression of the ASI mRNA. Single amino acids at a concentration of 10 mM were added to the amino acid-free NaKRB as a "fed" condition versus NaKRB alone to determine the amino acid specificity of repression for the ASI mRNA. The amino acids alanine, arginine, glycine, histidine, lysine, and proline were chosen for two reasons. First, included in the group are some of the amino acids that are known to have repressive activity on adaptive induction of System A-mediated transport (Kilberg et al., 1985). Second, all 20 amino acids were tested in a series of preliminary experiments, and a few amino acids showed no effect on the induction of the ASI mRNA; others had the ability to keep the ASI mRNA levels nearly fully repressed. We chose six amino acids and performed three independent Northern

analyses from three separate sets of cell cultures. The ASI mRNA abundance was quantitated by densitometry of the resulting autoradiographs. The ASI mRNA abundance in each amino acid-supplemented media, as compared to fed cells was: amino acid free, 2.10 ± 0.21; alanine, 1.28 ± 0.07; arginine, 1.28 ± 0.13; glycine, 2.00 ± 0.31; histidine, 1.48 ± 0.25; lysine, 1.99 ± 0.43; and proline, 2.23 ± 0.46. Most of the induction values fell between those for the fed (MEM) and the starved cells, although the induction in the presence of proline was at least as great as that seen for total starvation. Alanine and arginine proved to be the most effective repressors of the induction of the ASI mRNA, with only a 28% increase seen, compared to the nominal 100% to 125% increase seen in the starved condition.

Nuclear run-offs were conducted to determine if active transcription of the ASI gene changed during amino acid starvation. Nuclei from amino acid-fed and -starved cells were isolated as described in the methods section of this chapter, then incubated in the presence of α-[^{32}P]-UTP to synthesize radiolabelled RNA probe. This probe was used much like the first strand cDNA probes were used in differential hybridization, except in this case, the radiolabelled probe was hybridized to filter-bound cDNA inserts corresponding to the ASI and actin mRNAs. The

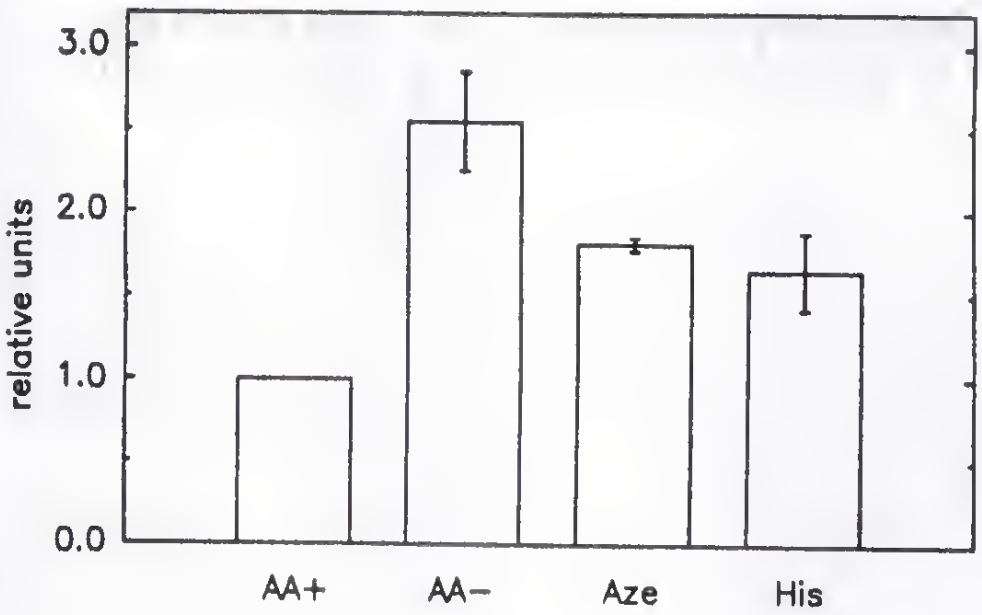

Fig. 3-9. Effect of the amino acid analogs L-azetidine-2-carboxylic acid and L-histidinol on ASI mRNA induction. Fao cells were cultured for 12 hours in MEM (AA+) or NaKRB (AA-) or in MEM with either of the inhibitors (Aze, His) added at 10 mM. After culture, total RNA was purified from the cells and used for Northern analysis. The data are from duplicate analyses quantitated by laser scanning densitometry and the AA+ condition was set to 1.0 to provide a standard of comparison.

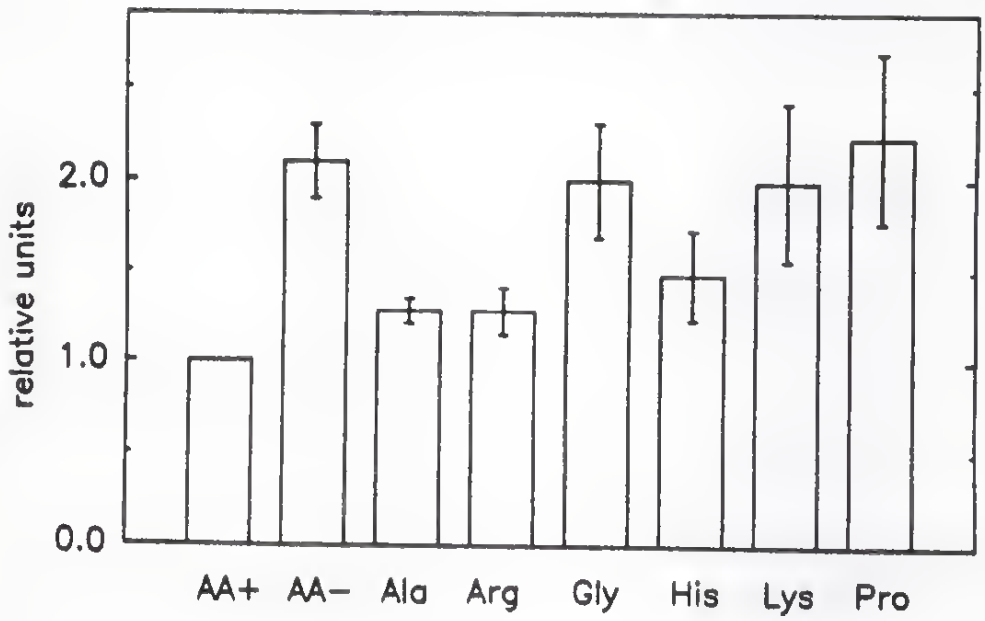

Fig. 3-10 Amino acid specificity of ASI mRNA repression in Fao cells. Hepatoma cells were cultured in amino acid-free medium (NaKRB) or NaKRB supplemented with a single amino acid at 10 mM. ASI mRNA levels were compared to the level in amino acid-starved or -fed Fao cells after 12 hours of culture with the amino acid. AA+, MEM; AA-, NaKRB alone; Ala, alanine; Arg, arginine; Gly, glycine; His, histidine; Lys, lysine; Pro, proline. Autoradiographs of Northern analyses were quantitated using laser scanning densitometry and the data represent averages of three separate experiments. The value for MEM was set to 1.0 as a standard for comparison.

amount of hybridization to the cDNA affixed to filters allowed an estimation of relative rates of active transcription of the two genes in both the amino acid-fed and -starved states. The relative rates of transcription were standardized to the rate of transcription determined at "time zero", that is, from cells not yet subjected to starvation. This "control" condition represents Fao cells growing in the presence of amino acids and serum. To begin an experiment, the culture medium was removed from the cells and replaced with NaKRB or MEM. Nuclei were isolated from cells at 0, 1.5, 3, 6, and 10 hours after the medium was changed. Radiolabelled RNA was made from the nuclei as described above, and an equal number of cpm, typically 5 x 10^6, were added to cDNA insert-bearing filters immersed in hybridization solution. Quantitation of the autoradiographs yielded the data presented in Figure 3-11. Transcription of actin mRNA decreased exponentially in both the fed and starved case, dropping to approximately 60% of the initial value after 10 hours in MEM, compared to a drop of greater than 75% in the amino acid-free NaKRB. The transcription of the ASI gene rose approximately 20% above its initial value at 1.5 hours in both MEM and NaKRB, then dropped exponentially in a manner similar to that seen with actin. The rate of transcription of the ASI mRNA after 10 hours of amino acid starvation is 50-75% of its initial value, compared to less than 25% for actin.

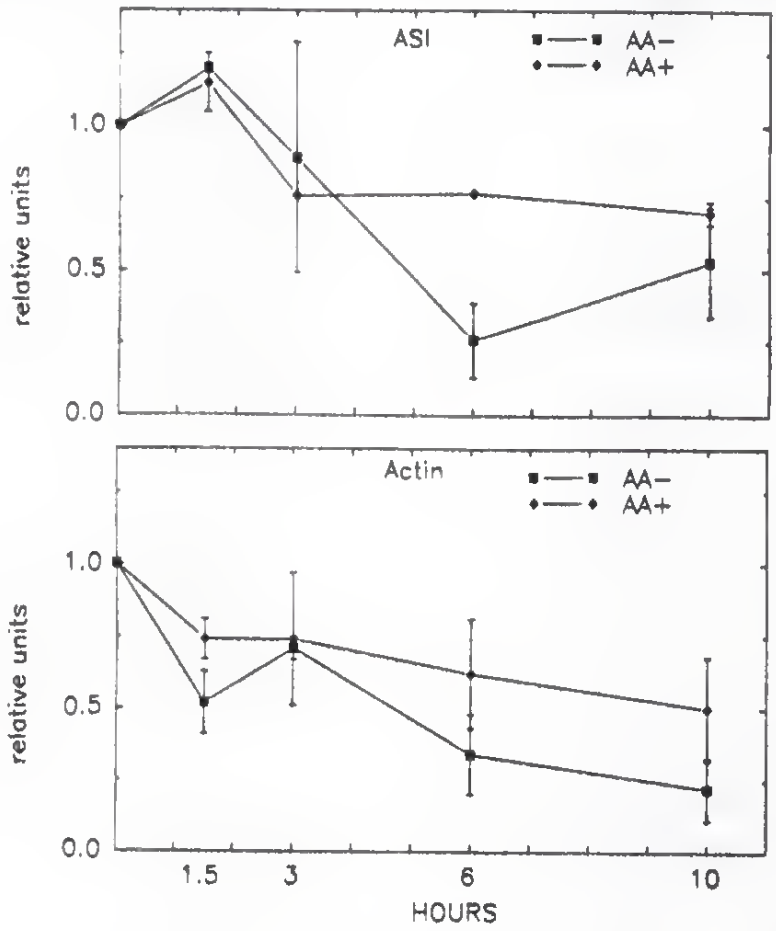

Fig. 3-11. Nuclear run-off assay for Fao cells incubated in amino acid-free or -supplemented medium. Active transcription of the ASI and actin genes in Fao cells was compared at 0, 1.5, 3, 6, and 10 hours of culture. Quantitation of the resulting autoradiographs following hybridization of [^{32}-P]-labelled RNA to filter-bound cDNA was by laser scanning densitometry. The data shown represent averages of two independent determinations for ASI and four determinations for actin. The results are consistent with those from various time points determined in ten other experiments.

Discussion

We have isolated one cDNA from our differential screening that corresponds to an amino acid-starvation induced (ASI) mRNA. We have quantified the induction of this mRNA to be between 2- and 3-fold in hepatoma cells starved for 9 to 12 hours. Although this induction may appear to be modest, the magnitude of induction is similar to many seen in other "plus-minus" studies (Fornace et al., 1988; Cox et al., 1988), especially at the earlier (3 to 6 hour) time points after the application of the stimulus. Furthermore, it was observed that while the ASI mRNA was increasing during this time period, several mRNAs that are reasonably stable in their expression, i.e., "housekeeping" genes, concurrently dropped considerably in abundance. For example, actin mRNA levels routinely dropped 50 to 75% during the same time period that the ASI mRNA abundance was increasing. Comparing the ratio of ASI mRNA to actin produced a relative ratio that routinely increased 5- to 10-fold during starvation. The repression of the actin mRNA responds quickly to the absence of amino acids with the response independent of the presence of fbs (data not shown). It is not clear if the repression of the actin mRNA is a direct response to the lack of amino acids or to a secondary cause, such as an amino acid-dependent exit from

active cell cycling. Schneider et al., (1988) presented results of a differential screening project, identifying 6 cDNA clones corresponding to mRNAs specifically expressed at growth arrest in NIH 3T3 cells. If the lack of amino acids is causing cells to shift into G_o, then the ASI clone may correspond to one of those 6 clones.

To verify the effect of amino acids on ASI mRNA abundance, several experiments were performed. It was found that the induction of ASI was partially repressible in a "refeeding" experiment (Fig. 3-5). A 10-fold induction of the actin mRNA was shown to occur when Fao cells were refed with amino acids after 12 hours of starvation. Presumably this rebound is part of the cellular response to provide more actin protein to a cell that may have been catabolizing the protein during starvation by autophagy (Poso et al., 1982). It is probably necessary for the cell to have a higher actin mRNA abundance during this recovery from starvation than would be seen in the "steady-state" condition.

The use of the metabolic inhibitors actinomycin D and cycloheximide showed that the induction of the ASI mRNA was dependent on both transcription and translation, pointing us toward models of regulation that involve either transcriptional activation of the ASI gene, decreased half-life of the ASI mRNA, or both in combination. The use of

the transcription inhibitor actinomycin D blocked the ASI induction and caused a decay in the amount of ASI mRNA present, while the translational inhibitor cycloheximide blocked the induction but maintained the ASI mRNA abundance at its initial level. The half-life of the ASI mRNA, as determined using actinomycin D, was approximately 7.5 hours in both the starved and fed cases. The actin mRNA, on the other hand, was seen to decay in a similar manner when cells were treated with either actinomycin or cycloheximide. The half-life of the actin mRNA was approximately 5.5 hours in MEM and 6.5 hours in NaKRB, although the difference between these numbers may be negligible, considering experimental error.

The analogs of proline and histidine, L-azetidine-2-carboxylic acid and L-histidinol, respectively, were used as an additive to amino acid supplemented media (Fig. 3-9). These analogs mimic starvation even while adequate levels of amino acids are present. We found that the ASI mRNA was induced above basal levels, but the induction was only about 50% of that seen in the amino acid-free situation. As these analogs are known to be strong inducers (5-fold or higher) of the heat shock response, we presume that ASI is not a member of the heat shock family. This was verified by Northern analysis of heat shocked Fao cells, which showed the ASI mRNA to remain uninduced (data not shown).

A second experiment was performed using six individual amino acids as potential repressors of ASI mRNA induction (Fig 3-10). The regulation of the ASI mRNA during these experiments lead us to propose that if the mRNA is not directly regulated by the presence or absence of amino acids, then the signal must be tightly linked to the drop in amino acid concentrations, such as "stalling" of the translational apparatus. This interpretation is supported by the amino acid analog studies that resulted in a partial induction of the ASI mRNA even in the presence of normal concentrations of amino acids. Other laboratories have reported amino acid-dependent proteolysis in hepatocytes (Poso et al., 1982) that parallels in many respects the induction/repression reported here of the ASI mRNA. The ability of individual amino acids to act as repressors of ASI mRNA induction did not parallel the use of only neutral amino acids in the original selection procedure. For example, alanine is a strong repressor of ASI and was used in the original amino acid-supplemented medium. However, the cation arginine is likewise a strong repressor, but was not included in the original mixture. This cationic involvement was unexpected and suggests a complex regulation not strictly related to the 6 neutral amino acids chosen as the original amino acid repressors.

Finally, active transcription of both the ASI and actin mRNAs were measured in amino acid-fed and -starved Fao cells using the method of nuclear run-offs to determine if the ASI gene could be transcriptionally regulated. It has become clear from these experiments that there is not a large activation of ASI gene transcription during starvation. A slight increase in the relative rate of ASI transcription was seen at 1.5 hours into the time course of the experiment. The increase was seen in both the starved and fed cases, and as the increase is very modest (15%), it is likely that the increase is not statistically significant, considering the large number of variables that can affect the results of the run-off experiments (Ausubel et al., 1987). Both actin and ASI transcription rates were seen to remain higher in the amino acid-fed case compared to the starved condition. This ratio of fed/starved transcription rates depended on obtaining equal amounts of radiolabelled RNA from nuclei for hybridizations. As less labelled probe was produced from starved cells than from fed cells, the use of equal amounts of probe for hybridizations probably results in an overestimation of the amount of newly transcribed RNA made by starved cells. Thus, although we found more transcriptional activity in fed compared to starved cells, the ratio between the two is probably underestimated. An alternative method to performing these

experiments would be to hybridize with radiolabelled probe made from equal numbers of cells, rather than using equal amounts of probe. Conventionally, the method of transcriptional analysis depends on the assumption of equal transcriptional activity for the variety of conditions assayed, and this is clearly not the case when comparing fed and starved cells.

In light of the data revealed by the transcriptional analysis, we believe the induction of the ASI mRNA is most likely due to decreased mRNA turnover during amino acid starvation, and this decrease, along with a near constant rate of transcription, allows the ASI mRNA to accumulate above its basal level. The involvement of a slight increase in transcription, difficult to detect by the nuclear run-off method may or may not be a contributing factor in the induction. The fact that both inhibitors actinomycin D and cycloheximide block the induction of the ASI mRNA during amino acid starvation leads us to speculate that another gene product is involved in the stabilization of the ASI mRNA during starvation. Precedence for such a model has recently been presented for the transferrin receptor mRNA, which has been shown to interact with a 90 kDa regulatory protein, allowing the turnover of this mRNA to be correlated to the availability of iron (Theil, 1990). In a similar fashion, there may be some response made by cells to

starvation, perhaps a stabilizing protein, that serves to protect a certain class of mRNAs from degradation during starvation. If this stabilizing factor was synthesized after the onset of starvation, it would explain why the ASI mRNA decay was identical in the amino acid-fed and -starved case when transcription was blocked by actinomycin D. It might be possible that only when the cell is free to transcribe genes and translate proteins that the stability of the ASI mRNA would change. Presumably, the actin mRNA, along with the other control mRNAs tested are not protected by this putative stabilizing agent.

It is clear that amino acids are playing some role in the regulation of the ASI mRNA abundance. It is unknown whether this regulation is direct, perhaps through the binding of amino acids to a regulatory protein, or a less direct control, for example regulation by availability of metabolic intermediates supplied by amino acids or removal of starved cells from active cell cycle. These questions may remain unanswered until more information is known about the ASI gene and the function of its gene product. We present data in Chapter 4 to characterize other aspects of the ASI mRNA and gene, to better understand this gene and begin an attempt to identify the function of the ASI gene product.

CHAPTER 4

CHARACTERIZATION OF A STARVATION-INDUCED mRNA

Introduction

The characterization of isolated genes usually begins with the determination of the DNA nucleotide sequence (Maxam and Gilbert, 1977; Sanger et el., 1977). Sequences stored in computer data bases, such as GenBank, allow comparison of newly determined sequences to those previously entered into the data bank. This comparison may allow newly determined sequences to be identified as a gene previously discovered, or alternatively, as a related DNA sequence with some partial homology to a previously entered sequence. A determination of the DNA sequence also allows one to search that sequence for potential polypeptide open reading frames (ORFs). This makes possible computer-generated predictions about the molecular size of the gene product. Computer-derived analyses predicting physical properties of the protein (Garnier et al., 1978; Kyte and Doolittle, 1982) may be performed to provide a preliminary model for a newly discovered gene product. These analyses are dependent on the isolation and determination of the complete coding sequence of the polypeptide, which necessitates the full-

length sequence being identified. When a new gene is identified by isolation of only a portion of the full-length cDNA, this necessitates a secondary screening of a cDNA library, such as lambda gt11 (Hunyh and Davis, 1985). Only a portion of a cDNA is necessary for use as a hybridization probe to allow a Southern blot to be performed (Southern, 1975). Southern analysis will allow preliminary information about gene structure to be determined, e.g., is the gene single copy or a repeated gene. Likewise, a partial cDNA fragment can be used to prepare probes for mRNA content by the blotting procedure known as Northern analysis (Thomas, 1980).

Further characterization of a newly discovered gene usually will include a comparison of the relative abundances of the corresponding mRNA in a variety of tissues. This information would be especially useful for a newly discovered gene product, because it would demonstrate if the mRNA was present in every tissue or not. The presence of an mRNA in every tissue might suggest the gene product is providing an essential cellular function, compared to a mRNA expressed in one or a few tissues. In this case, this might suggest that the protein performs some metabolic function unique to those tissues. Also, purification of total and membrane-bound RNA allows one to determine whether the site of translation for a mRNA is cytosolic or membrane-bound.

One of the aims of this work was to identify one or more amino acid-regulated mRNAs, and also determine if the System A amino acid transporter was represented as one of the mRNAs. We consider the identification of the ASI cDNA to be an important step in our work because the ASI mRNA appears to be the first mRNA detected in a mammalian system that is regulated by the absence of amino acids. The ASI cDNA and its corresponding gene should be important tools for use in investigating how mammalian cells cope with changes in metabolite or nutrient levels. The remainder of the data presented here was designed to gather information about the ASI cDNA, mRNA, and gene so that its cellular function and role in amino acid starvation may eventually be deduced.

The data presented in Chapter 4 focus on characterizing features of the ASI cDNA, mRNA, and gene. Information discussed in this chapter includes characterization of the length of the mRNA, the distribution of the ASI mRNA in rat tissues, relative abundance between rat liver and rat hepatoma cells, and the subcellular location of the ASI mRNA. The entire cDNA sequence is presented, from which the mRNA sequence may be derived. A putative ASI polypeptide is deduced from the cDNA sequence, and computer-aided analyses predicting physical characteristics of that polypeptide sequence are introduced. Synthetic ASI mRNA was made to

show that the mRNA could be translated in vitro to produce the putative ASI polypeptide. Finally, preliminary analyses of the ASI gene structure were performed in rat and a variety of other species, and some distinct differences were seen between murine and other eukaryotic groups.

Materials and Methods

Primer extension

We chose to use the method of Wallner et al., (1986) with some minor variations as suggested by Ausubel et al. (1987). A 30 bp sequence that was 40 bp from the 5' end of rat liver clone 5.8 (origin of this clone is introduced in the results section of this chapter) was chosen and a 30 base long oligodeoxynucleotide (MK03) was synthesized by the University of Florida DNA synthesis core facility (5'-CCGGAATGGCACACACTGCTTCTTTAAAGT-3'). This oligodeoxynucleotide, or primer, was radioactively labelled using [^{32}P]-dATP and the enzyme polynucleotide kinase. Ten picomoles of primer, 160 μCi of radiolabelled dATP, and 10 units of bacteriophage T4 polynucleotide kinase were mixed in a reaction buffer containing 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, and 100 μM sperimidine. The mixture was incubated for 10 minutes at 37°C to allow the end-labelling to occur followed by 2 minutes at 90°C to inactivate the enzyme. The mixture was then treated twice with ammonium acetate and ethanol (as described in Chapter 2) to separate

the unincorporated [^{32}P]-dATP from the precipitated labelled primer. The final pellet was dissolved in 100 µl of 0.3 M sodium acetate, pH 5.9.

Two micrograms of Fao mRNA were hybridized to 0.2 pmol of radiolabelled primer for 90 minutes at 65°C in a buffer containing 0.04 M PIPES, pH 6.4, 0.4 M NaCl, and 1 mM EDTA. After hybridization, the mRNA and primer was precipitated with sodium acetate and isopropanol followed by centrifugation at 13,000g for 15 minutes. The pellet was washed with 80% ethanol, air-dried, then dissolved in 25 µl of reverse transcription buffer containing 50 mM Tris, pH 8.0, 5 mM $MgCl_2$, 6 mM ß-mercaptoethanol, 50 mM KCl, and 2 mM each of dATP, dCTP, dGTP, and dTTP. To this volume were added 40 units of AMV reverse transcriptase (Seikagaiku) and 1 µl of RNase inhibitor "Inhibit-Ace" (5-Prime/3-Prime, manufacturer). The mixture was incubated for 90 minutes at 42°C to allow extension of the hybridized primers. After incubation, 100 µl of 2.5 M ammonium acetate were added and the mixture was phenol/chloroform extracted, the aqueous phase collected and precipitated with isopropanol as described earlier, followed by a 15 minute centrifugation at 13,000g. The pellet was rinsed with 80% ethanol, air-dried, then dissolved in 5 µl of TE buffer. Five microliters of formamide-containing stop buffer (see "DNA sequencing" below) were added, and after mixing, 3 µl of

this solution were subjected to electrophoresis next to a lane of known DNA sequence which was used as a molecular size standard. The two samples were size-fractionated on a 6% acrylamide/urea/TBE gel to determine the length of the extension product following autoradiography of the dried gel.

Preparation of RNA from rat tissues

RNA was prepared exactly as described in Chapter 2, with the following additional points to be noted. Animals were sacrificed and organs or tissue samples were immediately removed and flash frozen between two blocks of dry ice. After freezing all of the tissue types, RNA was prepared by mincing the frozen tissue into small pieces, followed by homogenization in denaturing guanidinium solution ("Solution D"), using approximately 5 ml of denaturing solution for each gram of tissue. Samples were homogenized in a glass mortice with a rotating teflon pestle powered by a benchtop drill press. Each sample was homogenized until all or most of the tissue had been dissolved into the denaturing solution. For some soft tissues, such as liver or brain, 2 or 3 strokes were sufficient, but tissues such as skeletal or heart muscle, 20 to 40 strokes were necessary to dissolve enough tissue to prepare adequate amounts of RNA. After preparing total RNA, we found it prudent to inspect the quality of the RNA made

by subjecting 2 μg of each sample to electrophoresis on a MOPS/formaldehyde/agarose minigel (80 V for 1 hour), as degradation of RNA is more likely to occur when preparing RNA from many tissues in a single animal. Upon confirmation of the integrity of the RNA, we proceeded to run larger amounts of RNA on larger gels for Northern blotting.

Purification of rat liver membrane-bound RNA

We followed the method of Mechler (1987) to prepare membrane-bound polysomes using isopycnic centrifugation in a discontinuous sucrose gradient. Thirty 15 cm culture dishes containing Fao cells grown to near confluence were placed on ice, the culture medium was then replaced with ice-cold PBS to interrupt translation. The cells were collected by scraping and pelleted with a brief 500g centrifugation. The cell pellet was homogenized in 8 ml of RSB buffer (RSB buffer is 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 7.4, and 10 mM vanadyl ribonucleoside complexes as a ribonuclease inhibitor) using a polytron homogenizer (Brinkman) on speed setting 5 for approximately 20 seconds. The homogenate was mixed with a 2.5 M sucrose solution made in TKM (TKM is 50 mM Tris, pH 7.4, 150 mM KCl, and 5 mM $MgCl_2$) to make the final sucrose concentration 2.1 M. A discontinuous gradient was set up as follows: 4 ml of 2.5 M sucrose/TKM were added to the bottom of a high-speed centrifuge tube. Then 10-20 ml of the 2.1 M sucrose/TKM homogenate solution

were added. Over the homogenate were layered 13 ml of 2.05 M sucrose/TKM followed by 6 ml of 1.3 M sucrose/TKM. The tubes were capped and centrifuged for 2 hours at 60,000 rpm in a Ti60 rotor (Beckman). The microsome fraction floated to the top of the tube during centrifugation and was collected with a sterile pipet. The 2-5 ml collected were then treated as a cell or tissue sample to prepare RNA using the method of Chomczynski and Sacchi (1987) as described in Chapter 2.

DNA sequencing

The method of enzymatic or "dideoxy" sequencing (Sanger, 1977) of cDNA was used to determine the DNA sequence of inserts in both double-stranded plasmids and single-stranded M13 clones. For double-stranded plasmids, the method of Kraft et al., (1988) was followed with some minor modifications. Plasmids purified using the Triton-lysozyme protocol were found to produce more uniformly readable sequence than plasmids purified by the alkaline lysis method (both procedures are described in Chapter 3 methods). We also found that separating the plasmid from co-purifying RNA by passage over a Sepharose-4B (Pharmacia) column was a relatively easy process yielding adequate amounts of plasmid, and that this plasmid preparation yielded DNA sequence that was far superior in terms of clarity and length of readable sequence when compared to

sequence obtained from unpurified plasmid (the description of the Sepharose-4B separation is described below). Five micrograms of double-stranded plasmid in 10 μl of distilled water were denatured prior to sequencing by adding 1 μl of 2 M NaOH, 2 mM EDTA and incubating the sample at 65°C for 2 minutes followed by two more minutes at room temperature. The plasmid was then precipitated by adding 5 μl of 1 M sodium acetate, pH 4.5, and 45 μl of ice-cold 100% ethanol. After mixing, the plasmid was incubated at -20°C for 20 minutes followed by centrifugation at 13,000g for 15 minutes. The plasmid DNA pellet was rinsed with 80% ethanol and dried for 5 minutes in a Savant "Speed-Vac". Sequencing was performed using Taq DNA polymerase purified from the heat tolerant bacterium Thermus aquaticus, and the "TaqTrack" DNA sequencing kit from Promega. The Taq DNA polymerase is functional at temperatures up to 95°C, allowing sequencing to be performed at temperatures of 70 to 95°C where secondary structure of DNA templates is reduced, permitting polymerization through highly structured regions. The higher temperature also increases the stringency of primer hybridization, which decreases non-specific binding of sequencing primers. Sequencing with Taq polymerase at 70°C was found to produce higher quality sequence than methods based on sequencing at 37 or 42°C. Two picomoles of sequencing primer were added to the denatured plasmid pellet

along with a 5X concentrated buffer making the final buffer concentration 50 mM Tris, pH 9.0, 10 mM $MgCl_2$, and dGTP, dCTP, and dTTP each at 7.5 μM. This mixture was allowed to incubate for 10 minutes at 37°C to allow the primer to anneal to the plasmid, followed by the addition of 2 μl of α-[$^{-35}$S]-dATP (1000 Ci/mmol) and 7.5 units of Taq polymerase. This mixture was then incubated at 37°C for 5 minutes, and was then split equally into four tubes containing 1 μl of the G, A, T, and C termination mixes. The composition of these mixes are as follows: G mix -- 50 μM ddGTP, 25 μM dGTP, 250 μM each of dATP, dCTP, and dTTP; A mix -- 350 μM ddATP, 25 μM dATP, 250 μM each of dCTP, dGTP, and dTTP; T mix -- 600 μM ddTTP, 25 μM dTTP, and 250 μM each of dATP, dCTP, and dGTP; C mix -- 160 μM ddCTP, 25 μM dCTP, and 250 μM each of dATP, dGTP, and dTTP. These mixes were incubated for 5 minutes at 70°C, then 4 μl of stop buffer containing 10 mM NaOH, 95% formamide, and 0.05% each of bromophenol blue and xylene cyanole were added. These sequencing reactions could be used either immediately for analysis by electrophoresis or stored for several days at -20°C. Samples were boiled for 5 minutes prior to loading 3 μl of each reaction on a 6% acrylamide/urea/TBE gel and subjected to electrophoresis at constant power of 52 watts. Gels were dried onto a sheet of Whatman 1mm paper without any fixation treatment, then used for autoradiography.

Sequencing of M13 clones was done identically with the following exception: approximately 2 μg of M13 DNA were added to 1 pmol of sequencing primer and allowed to hybridize for 10 minutes at 37°C. As M13 DNA is single-stranded, there is no need for a denaturation step. Although M13 cloning involves an extra step beyond routine cloning in double stranded vectors such as pUC or pGEM, in our hands the sequence of M13 clones was much less ambiguous.

Anchored polymerase chain reaction cloning

This method, introduced by Loh et al., (1989) and Frohman et al., (1988), was used to obtain the 5'-most end of the cDNA sequence of the ASI cDNA, following the method of Horlick et al., (1990). After primer extension was completed as described above, the primer extension product was excised, electroeluted from the gel, and then phenol/chloroform extracted and precipitated. This single-stranded DNA fragment was then "G-tailed" using dGTP and terminal deoxynucleotide transferase (TdT) as follows: the pellet from the electroelution was dissolved in 36 μl of distilled water, then added to 10 μl of 5X TdT buffer (5X buffer is 0.5 M potassium cacodylate, 10 mM $CoCl_2$, 1 mM DTT), 2 μl of 100 mM dGTP, and 25 units of TdT, mixed, and incubated at 37°C for 1 hour to add d(G) residues to the 3'-end of the DNA. The reaction was incubated for 10 minutes

at 70°C to inactivate the TdT enzyme. The DNA was ethanol precipitated and resuspended in 40 μl of TE buffer. This G-tailed single-stranded DNA was then used as the substrate for the anchored polymerase chain reaction (APCR) using the primer described previously and a second DNA primer, "HN14", (5'-AGGATCCAAGCTTGAATTCCCCCCCCCCC-3') obtained from the laboratory of Dr. H. S. Nick. This primer was used to anneal to the G-tailed 3'-end of the primer-extended cDNA as well as to provide the BamH I, Hind III, and EcoR I restriction sites in a "minilinker" region at the 5'-end of the cDNA PCR product. The PCR reaction was performed in 10 mM Tris, pH 8.3, 2.5 mM $MgCl_2$, 0.01% gelatin, 0.4 mM each of dATP, dGTP, dCTP, and dTTP, approximately 2 μg of each primer, and 10 ul of the tailed cDNA with 1 μl (5 units) of Taq DNA polymerase (Perkin Elmer/Cetus) in a final volume of 100 μl. This mixture was added to a 0.5 ml plastic microfuge tube, overlayed with mineral oil, then incubated as follows: 5 cycles of 1 minute at 94°C, 2 minutes at 25°C, and 3 minutes at 72°C which allows an initial low stringency annealing cycle. Subsequently, 23 higher stringency cycles of 1 minute at 94°C, 2 minutes at 62°C, and 3 minutes at 72°C, followed by an additional 7 minutes at 72°C were performed to increase the amount of DNA. The double-stranded cDNA produced by this reaction was purified by agarose gel electrophoresis followed by electroelution,

extraction and precipitation, and then blunt-ended using mung bean nuclease as follows: 10 µl of DNA were mixed with an equal volume of 2X mung bean nuclease buffer (2X buffer is 100 mM sodium acetate, pH 5.0, 60 mM NaCl, and 2 mM zinc sulfate) and 0.5 µl of mung bean nuclease (5 units). After mixing, the mixture was incubated for 30 minutes at 30°C, and the DNA was extracted with phenol/chloroform and ethanol precipitated. This blunt-ended, double-stranded cDNA was then ligated into the Sma I restriction site of the double-stranded RF form of the sequencing vectors M13mp18 and M13mp19. Potential positive clones, as determined by plating on X-gal/IPTG plates were grown up in 5 ml mini-preps. The M13 DNA was prepared, subjected to electrophoresis, blotted and hybridized to [^{32}P]-labelled ASI cDNA probe to identify false positives. Final validity of the clones came via sequencing, which produced the previously known 5'-end of the ASI cDNA followed by 143 bases of previously undetermined sequence of the 5'-most end of the ASI mRNA, with the G-tail and minilinker sequence directly thereafter. Once ligated into the M13 vectors, the cDNA could be sequenced and subcloned using the single- and double-stranded forms of M13, respectively.

Cloning of cDNA inserts into M13

We used the M13 sequencing vectors to subclone and sequence the ASI cDNA fragments of interest. cDNA inserts

were ligated into either the EcoR I or Sma I sites of M13mp18 and M13mp19 (Messing, 1983; Messing et al., 1977) as described in Chapter 3 for pUC based-plasmids. Ligations were used to transform competent JM109 cells and after transformation, cells were mixed with soft top LB or YT agar containing 0.7% (w/v) agar, 0.03% (w/v) X-gal, 0.3 mM IPTG, and 2 drops of exponentially growing JM109 cells. The molten top agar was then poured onto LB or YT agar (1.5% w/v) plates and grown overnight at 37°C. The colorless plaques, corresponding to cDNA insert-containing M13 phage, were pulled from the plate using a sterile glass capillary tube, added to 5 ml of liquid LB or YT broth with 2 drops of exponentially growing JM109 cells, and incubated with shaking overnight at 37°C. M13 phage, containing single-stranded DNA, were collected from the cleared supernatant by adding 0.2 volume of of a 20% polyethylene glycol-8000, 2 M NaCl solution, incubating on ice for 1 hour, and then centrifuging the mixture at 10,000g for 30 minutes. The phage was stored in a small volume of TE buffer, or phenol/chloroform extracted for hybridization analysis or sequencing.

Analysis of DNA sequence

DNA sequence was stored and analyzed using Microgenie software (Beckman). In addition to searching the GenBank data for DNA and amino acid sequence homologies to known

genes or proteins, the software allowed us to generate a predicted secondary structure of the ASI amino acid sequence using the algorithm of Garnier et al., (1978). The Garnier algorithm is as effective as other algorithms currently used for such analyses, predicting the conformation of about 50% of the amino acids correctly. It is better suited than some of the others for computer-aided computation. A hydrophobicity/hydrophilicity plot of the putative protein using the algorithm of Kyte and Doolittle (1982) was also performed.

In vitro transcription

The synthesis of 5'-capped RNA transcripts in vitro was accomplished following a protocol supplied by the Promega Corporation. The cDNA insert containing the entire coding sequence for the ASI-1 protein was subcloned into a pGEM-9Z plasmid vector (Promega) which has both the T7 and SP6 RNA polymerase promoters flanking the polylinker site in the plasmid. The two promoters are arranged in opposite orientations, to allow transcription of the insert in both the sense and anti-sense directions. Five micrograms of the insert-containing plasmid were incubated in a final volume of 50 μl at 37°C for 60 minutes in 40 mM Tris, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.1 mg/ml BSA, 20 units "RNasin" ribonuclease inhibitor (Promega), 0.5 mM each of ATP, CTP, and UTP, 50 μM GTP, 0.5 mM GpppG, and

40 units of SP6 or T7 RNA polymerase. After incubation for one hour, the mixture was extracted with phenol:chloroform once followed by chloroform alone, and then the RNA was precipitated with ethanol and recovered by centrifugation.

In vitro translation

The rabbit reticulocyte lysate in vitro translation system (Promega) was used to translate synthetic RNA molecules. Incubations were performed as follows: two to five micrograms of RNA were added to 35 μl of nuclease treated lysate along with 7 μl H_2O, 40 units of "RNasin" ribonuclease inhibitor, 1 μl of a mix of amino acids (except methionine) at 1 mM each, and 40 μCi of [^{35}S]-methionine in a final volume of 50 μl. The mixtures were allowed to incubate for 1 hour at 30°C, then analyzed using SDS-PAGE.

Southern blotting

We used modifications of the methods of Southern, (1975) and Smith and Summers, (1980) as well as methods presented by a variety of manufacturers to arrive at a protocol that produced uniformly good results. We used the method presented in Davis et al., (1986) to prepare genomic DNA from both cells in culture and tissue samples. Briefly, tissue or cell pellets (0.1 - 0.2 g) were homogenized with a teflon pestle in 2 ml of 0.1 M NaCl, 0.2 M sucrose, 0.01 M EDTA, and 0.3 M Tris, pH 8.0 until the mixture was devoid of large tissue chunks. After 0.1 volume of 10% SDS was added

and mixed by vortexing, the mixture was incubated for 30 minutes at 65°C. A 0.3 volume aliquot of 8 M sodium acetate was added and mixed, and then incubated on ice for 1 hour. The mixture was centrifuged at 5000g for 10 minutes and the supernatant fraction collected. This volume was then extracted one time with phenol/chloroform, followed by an extraction with chloroform alone. The genomic DNA was then precipitated with three volumes of ethanol and collected by centrifugation. After preparing the DNA as outlined, the DNA was subjected to two additional phenol/chloroform extractions to further purify the DNA from protein contaminants. Samples were digested overnight in 20 µl containing 10 µg of DNA and at least 20 units of the appropriate restriction endonuclease. Digests were performed at 37°C, unless the optimal temperature for the enzyme was different, such as Taq I (65°C). Restricted DNA samples were size-fractionated on 1% agarose gels using a 1X TAE buffer (1X TAE is 40 mM Tris, pH 7.2, 20 mM sodium acetate, 1 mM EDTA), typically run overnight at 30 V with tank buffer recirculated during the run. After electrophoresis, the gel was removed from the tank and stained with ethidium bromide to visualize the DNA under UV light. The gel was photographed to document the location of molecular size standards, then prepared for blotting by shaking gently in denaturing solution (0.5 M NaOH, 1.5 M

NaCl) for 20 minutes, followed by 30 minutes in neutralizing solution (1 M Tris, pH 8.0, 1.5 M NaCl). The positively charge nylon membrane Zeta-bind (AMF) was used for blotting and was soaked for 2 minutes in distilled water followed by 20 minutes in 10X SSC (1X SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0). At this point, the DNA was transferred to the membrane using capillary action provided by stacking paper towels above the membrane. Blotting was completed overnight, and transfer verified by inspecting the blot and gel on a UV light source for ethidium bromide-stained DNA. After transfer, the blot was washed for 30 minutes in 2X SSC, then thoroughly dried either overnight at room temperature or for 1 hour at 80°C. Blots were hybridized to [^{32}P]-labelled DNA probes using the methods described in Chapter 3. Blots were washed at high stringency, using several changes of 0.1X SSC containing 0.1% SDS for a total of 1 hour at 65°C. Washed blots were evaluated by autoradiography with Kodak XAR film with intensifying screens, with typical exposure times of 1 to 3 days.

Results

In order to characterize fully the ASI mRNA and its corresponding gene, a series of experiments were conducted to obtain more information about ASI. A sample of Fao RNA was size-fractionated on a 1.5% agarose MOPS/formaldehyde gel along with RNA size standards to try to estimate the

length of the ASI mRNA. Although we had previously used 1.0% agarose gels for our Northern analyses, 1.5% was used for this particular analysis because it provides better resolution for smaller RNAs (< 1 kb). From Northern analyses (Chapter 3), we knew the approximate size of the ASI mRNA was less than 1 kb. After size-fractionating the two samples, the gel was blotted and hybridization was performed with [^{32}P]-labelled ASI and ß-actin cDNA probes. The blot was washed as described in Chapter 3 and used for autoradiography (Figure 4-1). We estimated the size of the mRNA to be 600 bp, with an error of ± 50 bp.

The most precise method for analyzing the size of a mRNA is the method of primer extension, which allows the 5'-end of an mRNA to be located with certainty to the nearest nucleotide (McKnight and Kingsbury, 1982). In addition, methods utilizing the polymerase chain reaction (PCR) and Taq DNA polymerase allow the cDNA cloning of these 5'-most regions if a full-length cDNA is not obtained by library screening. Use of a primer-extended cDNA product allowed us to produce the complete ASI cDNA using the "anchored-PCR" (APCR) method (Frohman et al. 1988; Loh et al. 1989), when the result of the initial primer extension analysis made it clear that our cDNA clones were not full length.

As described in the methods section above, an oligonucleotide was synthesized complementary to the

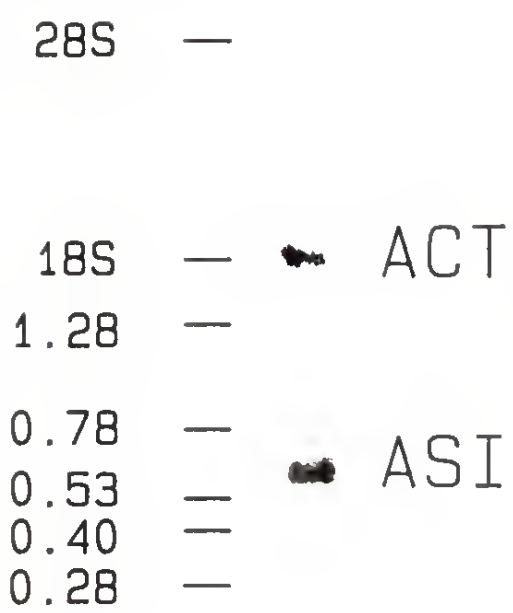

Figure 4-1. Molecular size determination of the ASI mRNA. Northern analysis was performed to compare the length of the ASI mRNA to a set of known mRNA standards. Total cellular rat Fao hepatoma RNA was size-fractionated along with the molecular standards on a 1.5% agarose MOPS/formaldehyde gel, blotted and hybridized to [^{32}P]-labelled ASI and ß-actin cDNA probes. Size standards given on left, ASI: location of ASI mRNA, ACT: location of ß-actin mRNA.

single-stranded ASI mRNA. This sequence was 38 bp from the 5'-most end of normal rat liver cDNA clone 5.8, which contained more 5'-end sequence than any of the other ASI rat liver cDNA clones identified. After hybridization of the 30-mer to rat hepatoma Fao mRNA, the primer was extended using the RNA-dependent DNA polymerase, AMV reverse transcriptase, as described previously. The primer extension reaction was then analyzed on a 6% acrylamide/urea sequencing gel with a parallel lane of a known sequencing reaction to serve as a molecular size standard. The gel was dried and analyzed by autoradiography (Figure 4-2). The primer extension product was 211 bases long, and as we started the primer extension reaction 68 bases within our 5'-most cloned fragment, we inferred that the 5'-end of the mRNA must extend 143 bases in the 5'-direction beyond the rat liver clone 5.8. This would then make the length of the complete hepatoma ASI mRNA 612 bp long, plus the length of the 3'-end poly(A) tail. The 211 base-long primer extension product was electroeluted from the gel and used as the substrate for the APCR reaction. The fragment was dGTP-tailed at the 3'-end and mixed with two oligonucleotide primers and Taq polymerase, and subjected to 28 cycles of annealing, extension, and denaturation to produce a double-stranded cDNA corresponding to the 5'-most end of the ASI mRNA. The reaction product was analyzed on a 2% agarose/TBE

gel, running nearly identically with a 242 bp molecular size standard. This gel fragment was electroeluted, blunt-ended with mung bean nuclease, and then ligated into the Sma I restriction site of both M13mp18 and M13mp19 for sequence analysis.

Along with the determination of the ASI mRNA length, we chose to do three additional series of experiments to further characterize the ASI mRNA, a tissue distribution, a comparison between normal liver and hepatoma tissue, and subcellular location of mRNA translation. Total RNA from 11 different rat tissues was prepared and used for Northern analysis. The result of one of several analyses performed is shown in Figure 4-4. The ASI mRNA was shown to be present in every rat tissue tested. It appeared to be most abundant in adipose, lung, spleen, and testes, compared to whole blood, kidney, and liver which are the tissues in which ASI was least abundant. The ASI mRNA appeared to be the same length in every tissue tested. As the abundance of the ASI mRNA was the least in liver, we wanted to compare the ASI abundance in liver and in the Fao cell line, in which ASI appeared to be a relatively abundant mRNA. This was done by subjecting a variety of Fao RNA amounts to Northern analysis along with one 15 μg sample of rat liver RNA. The autoradiograph from this analysis is shown in Figure 4-5. Laser scanning densitometry of the

Figure 4-2. Primer extension of the ASI mRNA. A 30 residue synthetic oligodeoxynucleotide complementary to the ASI mRNA 38-68 bp from the 5'-end of the then partial ASI mRNA sequence was used as a primer for AMV reverse transcriptase. After allowing the [^{32}P]-labelled primer to hybridize to Fao mRNA, it was extended with AMV reverse transcriptase. The reaction product was subjected to electrophoresis on a 6% acrylamide gel, the gel dried and used for autoradiography. P: primer extension reaction, M: molecular size standards. Length of primer extension product was 211 nucleotides.

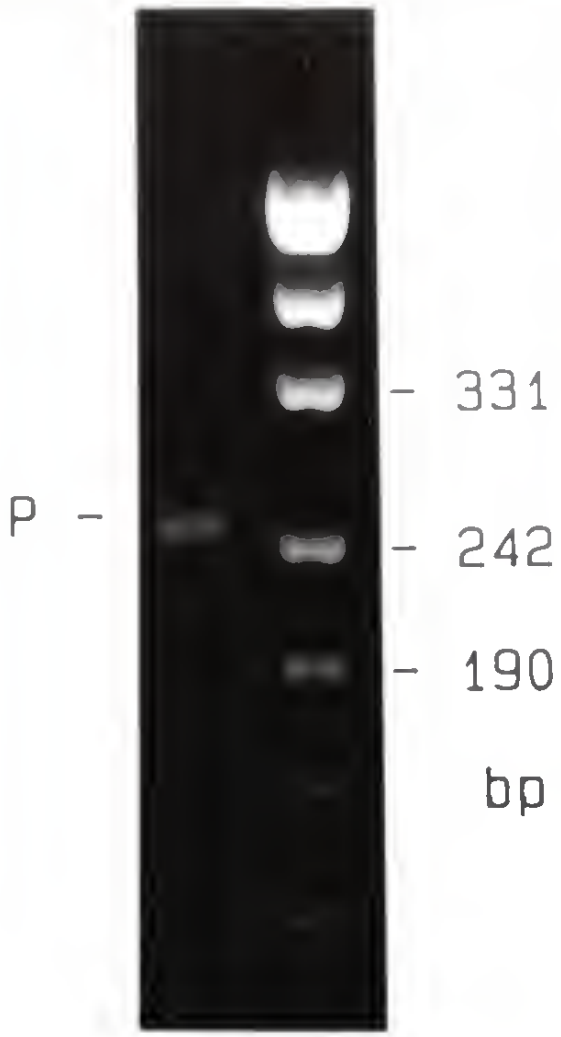

Figure 4-3. Anchored polymerase chain reaction. To prepare double-stranded DNA corresponding to the then undetermined 5'-region of the ASI mRNA, the APCR method was employed using two synthetic oligodeoxynucleotide primers and Taq DNA polymerase. The APCR reaction was analyzed on a 2% agarose/TBE gel. Lane 1, APCR reaction; lane 2, molecular size standards. P: PCR product. Molecular sizes flanking APCR product are shown.

autoradiograph determined the relative abundance of the ASI mRNA to be approximately 7-fold higher in Fao mRNA than in rat liver.

As mRNAs are translated on free, cytosol-located ribosomes for cytosolic, mitochondrial, or nuclear proteins versus those synthesized on endoplasmic reticulum (ER)-bound ribosomes for proteins destined to be integral ER, golgi, or plasma membrane or secreted proteins, we chose to analyze RNA purified from the rough endoplasmic reticulum in an attempt to characterize the ASI mRNA as being a mRNA translated on either "free" or "bound" ribosomes. Membrane-bound RNA was purified (Mechler, 1987), and used in comparison with total cellular RNA made by the method of Chomczynski and Sacchi (1987). These pairs of samples were used for Northern analyses and the blots were probed by ß-actin, a known cytosolically-translated mRNA, rat serum albumin (RSA), a mRNA known to be translated by membrane-bound ribosomes, and ASI. Analysis with actin yielded strong hybridization to the total RNA sample, with only slight hybridization to the membrane-bound, probably representing some contamination of the membrane-bound RNA with cytosolic RNA. With the RSA probe, the pattern was the opposite, the strongest hybridization being in the membrane-bound lane, as purification of this class of RNA increased the relative abundance of the membrane-bound mRNA

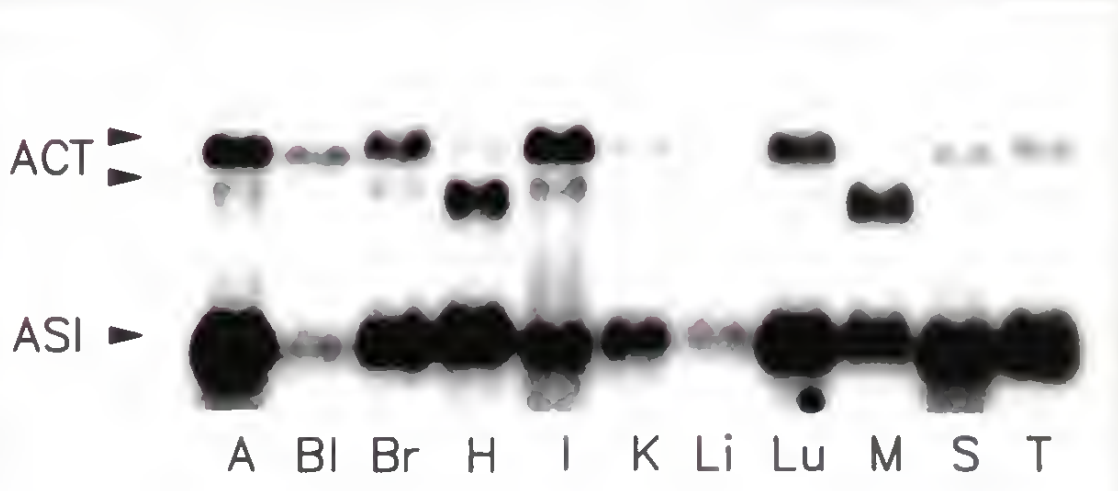

Figure 4-4. Rat tissue distribution of ASI mRNA. Total cellular RNA was prepared from tissues of a male Sprague-Dawley rat, size-fractionated by electrophoresis, blotted and subjected to Northern analysis. Each lane represents 10 μg of total RNA per tissue. Equivalent loading of lanes was confirmed by densitometric scanning of a photographic negative of ethidium bromide-stained blot (not shown). All lanes contain equivalent amounts of RNA +/- 10%. A: adipose, Bl: whole blood, Br: brain, H: heart, I: small intestine, K: kidney, Li: liver, Lu: lung, M: skeletal muscle, S: spleen, T: testes. ASI mRNA and actin (ACT) mRNAs (2 isoforms) are marked with arrows.

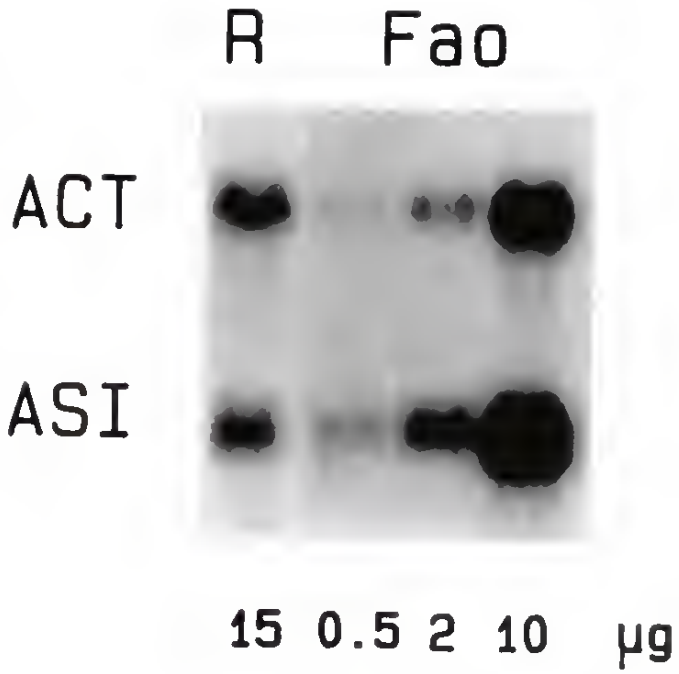

Figure 4-5. Relative abundance of the ASI mRNA in rat Fao hepatoma cells and normal rat liver. Relative abundance of ASI mRNA was compared by electrophoresis followed by Northern analysis and autoradiography. Autoradiograph compares relative abundance of ASI mRNA in 15 µg of rat liver total RNA (R) in lane 1, and 0.5, 2.0, and 10 µg of Fao total RNA in lanes 2-4. ACT: actin mRNA; ASI: ASI mRNA.

proportional to the degree of purification. The ASI mRNA followed the exact same pattern of hybridization as actin, and we infer from this result that the ASI mRNA is synthesized on cytosolic or "free" ribosomes (Figure 4-6).

Another way to obtain potentially useful information from a cDNA clone is to analyze the DNA sequence. We determined the sequence of the 409 bp ASI cDNA and found several features that suggested that the cDNA did indeed correspond to a polypeptide-encoding mRNA. First of all, there was a stretch of 17 d(A) residues at one end of the cDNA, which we interpreted as the remnant of the poly(A) tail of the ASI mRNA; hence, this end of the cDNA corresponded to the 3'-end of the mRNA. This assumption was strengthened by the fact that just prior to the poly(A) stretch were two potential polyadenylation signal sequences (AATAAA) 12 and 27 bp prior to the poly(A) sequence itself. Prior to the polyadenylation signal sequences was a long open reading frame followed by a stop codon, the stop codon actually being within the first polyadenylation signal sequence. We presumed that the clone did not contain a complete amino acid-coding sequence because the open reading frame continued to the 5'-end of the cDNA insert and did not begin with a start codon. Also another consideration was the fact that our 409 bp cDNA clone was somewhat shorter than our 600 bp estimate of the ASI mRNA length determined

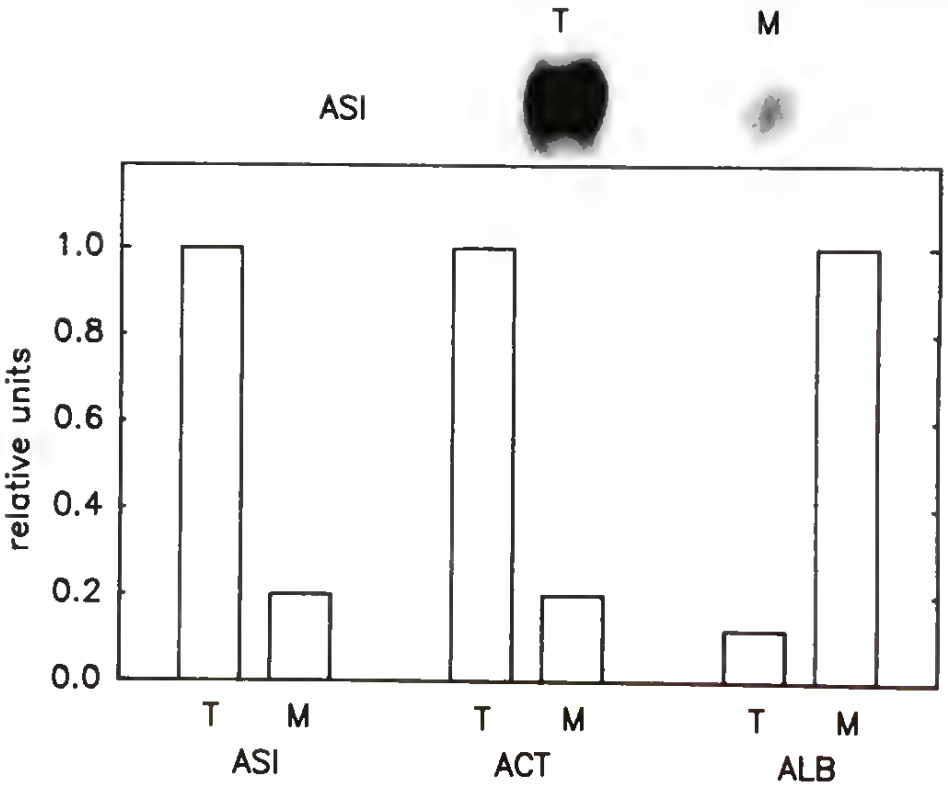

Figure 4-6. Localization of ASI mRNA to cytoplasmic ribosomes in rat liver. Total RNA and membrane-bound RNA was purified from rat liver, subjected to electrophoresis, blotted, and used for Northern analysis. Top: autoradiograph of ASI mRNA showing relative abundance in total (T) and membrane-bound (M) fractions, using 10 μg of RNA per lane. Bottom: quantitation of relative abundances of the ASI, ß-actin (ACT), and albumin (ALB) mRNAs in the total and membrane-bound fractions.

from Northern analysis. The sequence of the 409 bp cDNA insert is included in Figure 4-8 as the last 409 bp of DNA sequence. We attempted to isolate the full length ASI cDNA by using the 409 bp insert as a probe to screen a lambda gt11 rat liver cDNA library (Clontech), using library plating and probe preparation methods as described in Chapters 2 and 3. We screened 300,000 plaques from this library, obtained approximately 300 potential positive clones, and purified 10 of these clones. These ten clones were termed R5.1 through R5.10 to signify their rat liver library origin ("R") and their relationship to the original clone S-5 ("5"). After purifying these clones, phage DNA was prepared as described, and EcoR I cDNA inserts from the clones were analyzed by gel electrophoresis. The inserts ranged from 300 bp to 450 bp in length. We sequenced the clones to determine the relationship between these clones and the ASI 409 bp insert. Clone 5.8 provided us with new DNA sequence 5' to the ASI 409 bp cDNA. The cDNA insert from clone 5.8 was 392 bp long, and extended 77 bp beyond the 5'-end of the ASI 409 bp cDNA. After analyzing the sequence of R5.8, we determined that the open reading frame found in the ASI cDNA extended those additional 77 bases in the 5'-direction but did not include a start codon. This result lead us to believe that we had detected neither the 5'-end of the full length sequence nor the beginning of the

polypeptide coding sequence. At this point, the APCR method described in the Methods section was used to clone the 5'-most region of the ASI cDNA. We isolated an additional 143 bp of ASI cDNA that was 5' to both the original 409 bp as well as clone R5.8. The primer extension analysis showed us that this 143 bp represented the 5'-most end of the ASI cDNA. The complete ASI cDNA sequence was found to be 632 bp long with a potential polypeptide open reading frame of 562 bp, or 184 amino acids long (Figure 4-8). The putative amino acid sequence of the ASI polypeptide is included with the cDNA sequence (Figure 4-8), and again separately in Figure 4-10. The 184 residue polypeptide has a predicted molecular weight of 21.4 kDa. A summary of the DNA fragments sequenced to obtain the final full length ASI cDNA sequence is shown in Figure 4-7. Figure 4-9 shows the potential open reading frames in the three different potential reading frames of the cDNA sequence. As diagrammed, the first 2 reading frames have only a few potential open reading frames, the longest of these being approximately 10 amino acids long. Reading frame three, on the other hand, has a 562 bp or 184 residue reading frame. Modifications of the algorithms of Staden, (1984) and Gribskov et al., (1984), a part of Microgenie DNA analysis software (Beckman), was used to predict the probability of this reading frame encoding a polypeptide, based on codon

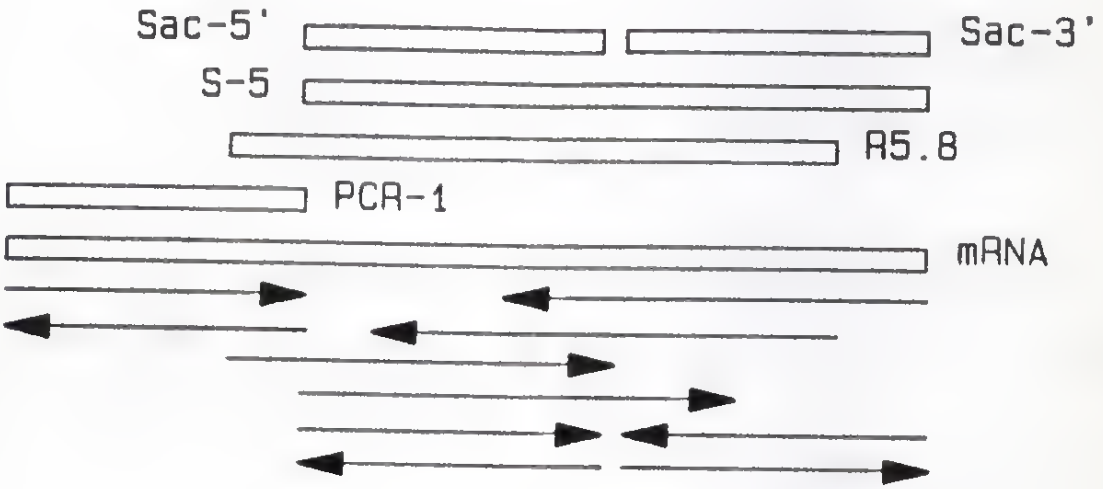

Figure 4-7. Sequencing strategy of the ASI cDNA. Horizontal bars indicate cDNA fragments derived from the ASI mRNA. cDNA fragments sequenced were clone S-5, 2 derivatives of the S-5 cDNA produced by Sac I digestion ("Sac-5'" and "Sac-3'"), clone R5.8 from rat liver, and the APCR cDNA fragment, PCR-1. Arrows below the fragments indicate length and direction of sequencing reactions performed to compile full length cDNA sequence corresponding to the ASI mRNA. Sequence reactions were performed using Taq DNA polymerase.

```
1          CTGTTAGCGGCCAGAGGTGACCTGTGAAGATGGTTCGCTACTCCCTT
                                          MetValArgTyrSerLeu

48   GACCCAGAAAACCCCACGAAATCATGCAAGTCAAGAGGCTCAAACCTTCGTGTT
     AspProGluAsnProThrLysSerCysLysSerArgGlySerAsnLeuArgVal

102  CACTTTAAGAACACCCGGGAAACTGCCCAGGCCATCAAGGGTATGCATATCCGC
     HisPheLysAsnThrArgGluThrAlaGlnAlaIleLysGlyMetHisIleArg

156  AAAGCCACCAAGTATCTGAAGGATGTCACTTTAAAGAAGCAGTGTGTGCCATTC
     LysAlaThrLysTyrLeuLysAspValThrLeuLysLysGlnCysValProPhe

210  CGGCGGTATAACGGTGGAGTTGGTAGGTGCGCCCAGGCCAAACAGTGGGGCTGG
     ArgArgTyrAsnGlyGlyValGlyArgCysAlaGlnAlaLysGlnTrpGlyTrp

264  ACACAGGGACGGTGGCCAAAAAAGAGTGCTGAATTTTTGCTGCACATGCTTAAA
     ThrGlnGlyArgTrpProLysLysSerAlaGluPheLeuLeuHisMetLeuLys

318  AATGCAGAGAGTAATGCTGAACTTAAGGGTTTGGATGTAGACTCTCTGGTCATT
     AsnAlaGluSerAsnAlaGluLeuLysGlyLeuAspValAspSerLeuValIle

372  GAACACATCCAGGTGAACAAGGCTCCTAAGATGCGCAGACGGACCTACAGAGCT
     GluHisIleGlnValAsnLysAlaProLysMetArgArgArgThrTyrArgAla

426  CACGGCCGGATTAACCCATACATGAGCTCCCCCTGCCACATCGAAATGATCCTC
     HisGlyArgIleAsnProTyrMetSerSerProCysHisIleGluMetIleLeu

480  ACTGAGAAGGAACAGATTGTTCCAAAGCCAGAAGAGGAGGTTGCACAGAAGAAA
     ThrGluLysGluGlnIleValProLysProGluGluGluValAlaGlnLysLys

534  AAGATATCCCAGAAGAAATTGAAGAAACAAAAGCTCATGGCACGGGAATAAATT
     LysIleSerGlnLysLysLeuLysLysGlnLysLeuMetAlaArgGluEnd

588  CAGCATAAATAAATGCGGATAAAGTAAAAAAAAAAAAAAAAAAA
```

Figure 4-8. ASI cDNA and putative protein sequence. Complete cDNA sequence of 632 bp corresponding to the ASI mRNA. Sequence was obtained by enzymatic sequencing of cDNA clones obtained from Fao and rat liver gt11 libraries as well as cDNA prepared using the APCR method. cDNA sequence includes a putative open reading frame (30-581), two polyadenylation signal sequences (underlined), and remnants of the poly(A) tail of the ASI mRNA (612-632).

preference exhibited by actual coding regions (data not shown). The algorithm predicts that most of the putative ASI amino acid sequence follows patterns of codon usage in known proteins, strengthening our confidence that the putative open reading frame of the ASI cDNA is actually translated into a polypeptide.

The predicted secondary structure of the ASI polypeptide is presented in Figure 4-11, as determined using the algorithm of Garnier et al., (1978). Our model for the polypeptide includes several prominent α-helical regions and short connecting regions of random coil and ß-sheet patterns. A hydrophilic plot of the ASI polypeptide was also prepared using the Microgenie software, and is displayed in Figure 4-12. The polypeptide is mostly hydrophilic, with only four short regions exhibiting appreciable hydrophobicity.

To show the protein coding potential of the ASI cDNA clone, a near-full length ASI cDNA was subcloned into the EcoR I and Hind III restriction sites of the pGEM-9Z plasmid (Promega). The ASI cDNA used for this procedure was prepared using a PCR reaction. Two 30-base long oligonucleotides were synthesized for this purpose, one each from the 5'- and 3'-ends of the ASI cDNA sequence. The 5'-end primer was 5'-TGTTAGCGGCCAGAGGTGACCTGTGAAGAT-3', and the 3'-end primer 5'-ATGCTGAATTTATTCCCGTGCCATGAGCTT-3'.

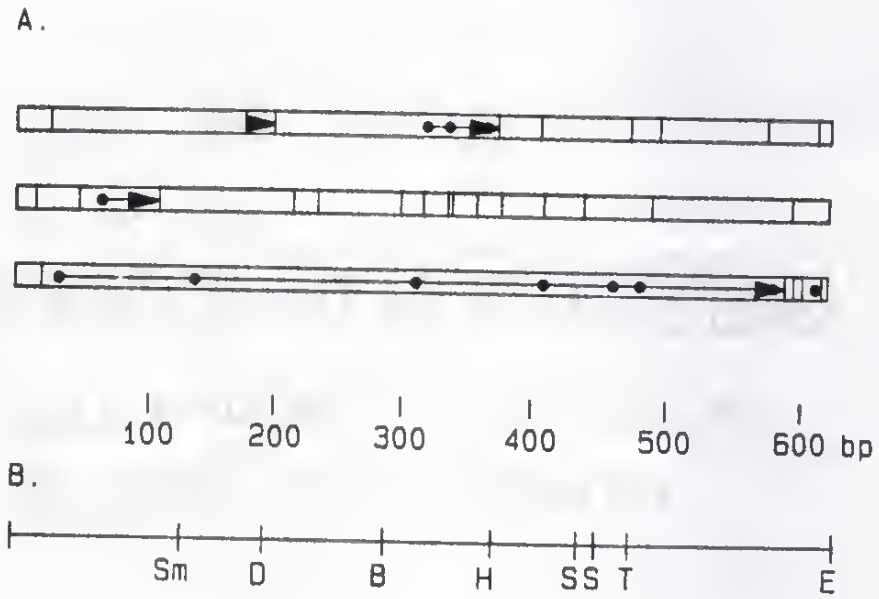

Figure 4-9. Map of the rat ASI cDNA sequence.
A. Potential open reading frames (ORFs) in the ASI cDNA sequence. Start and stop codons plotted in all three potential reading frames. Methionine residues, representing potential start sites are represented as circles, translation stop codons as vertical lines. Potential open reading frames are drawn in as an arrow from methionine residues to stop codons. B. Restrction endonuclease map of the ASI cDNA. Restrction sites are marked with a vertical line at their location on the cDNA. Sites are labelled with the appropriate restriction enzyme abbreviation: Sm, Sma I; D, Dra I; B, Bgl I; H, Hae III; S, Sac I; T, Taq I, E, EcoR I.

```
1    MetValArgTyrSerLeuAspProGluAsnProThrLysSerCysLysSerArg
19   GlySerAsnLeuArgValHisPheLysAsnThrArgGluThrAlaGlnAlaIle
37   LysGlyMetHisIleArgLysAlaThrLysTyrLeuLysAspValThrLeuLys
55   LysGlnCysValProPheArgArgTyrAsnGlyGlyValGlyArgCysAlaGln
73   AlaLysGlnTrpGlyTrpThrGlnGlyArgTrpProLysLysSerAlaGluPhe
91   LeuLeuHisMetLeuLysAsnAlaGluSerAsnAlaGluLeuLysGlyLeuAsp
109  ValAspSerLeuValIleGluHisIleGlnValAsnLysAlaProLysMetArg
127  ArgArgThrTyrArgAlaHisGlyArgIleAsnProTyrMetSerSerProCys
145  HisIleGluMetIleLeuThrGluLysGluGlnIleValProLysProGluGlu
163  GluValAlaGlnLysLysLysIleSerGlnLysLysLeuLysLysGlnLysLeu
181  MetAlaArgGluEnd
```

Figure 4-10. Predicted amino acid sequence of the rat ASI protein. Polypeptide sequence results from the translation of the 562 bp open reading frame (Figure 4-9) into a 184 residue long amino acid sequence.

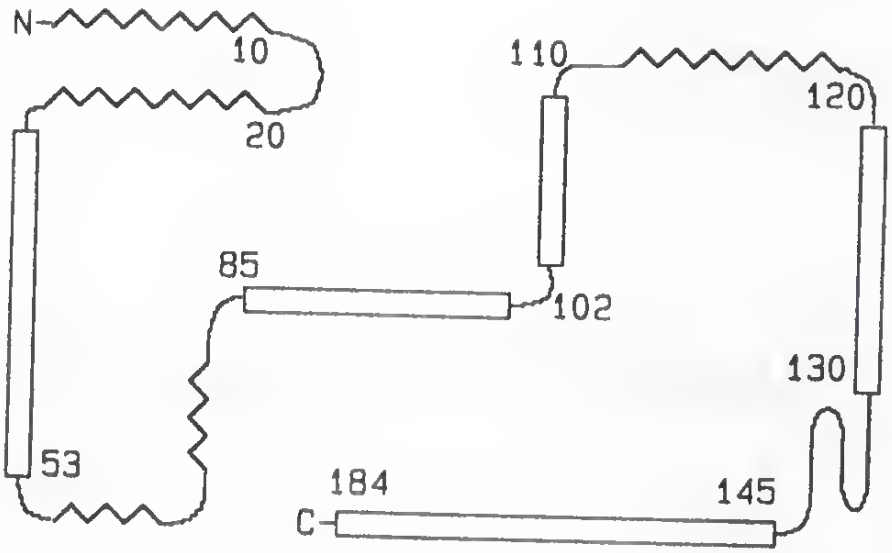

Figure 4-11. Predicted secondary structure of the ASI protein. The secondary structure of the 184 residue long rat ASI protein was predicted using the algorithm of Garnier et al. (1978). Sawteeth, ß-sheet; rectangles, α-helix; solid lines, random coil and turns.

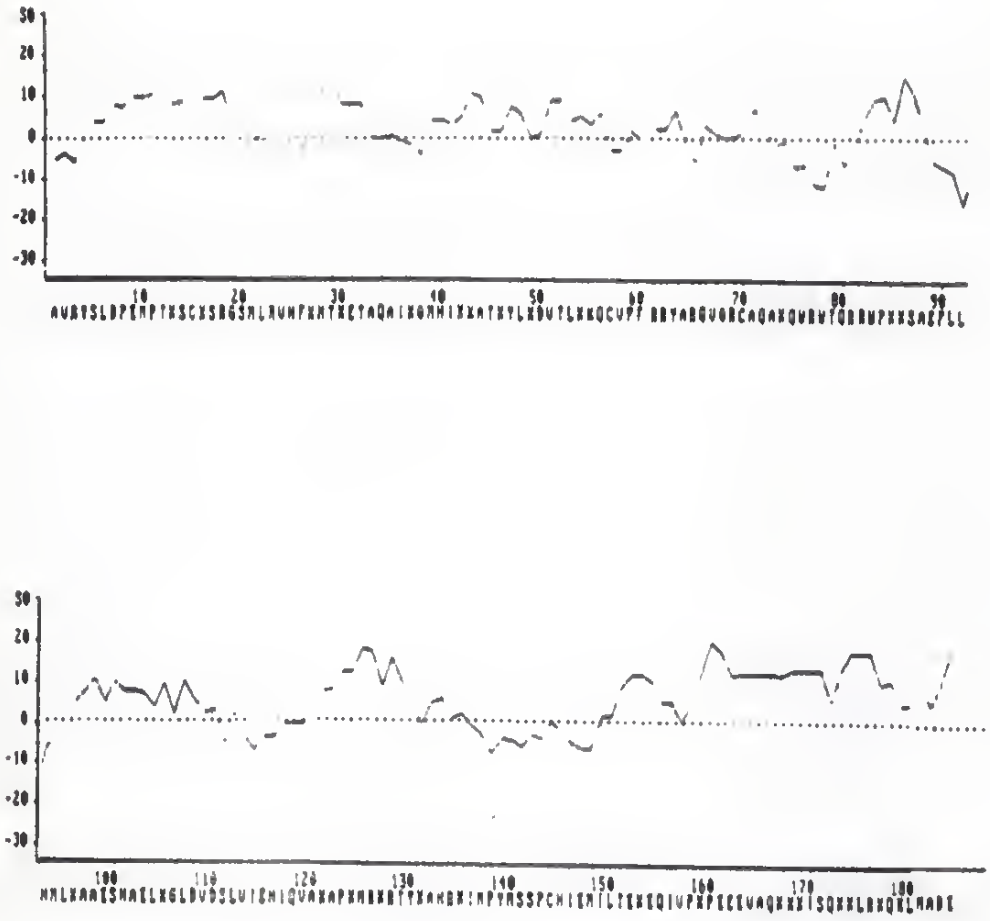

Figure 4-12. Predicted hydrophilic plot of the rat ASI protein. Hydrophilicity was determined by the method of Kyte and Doolittle (1982) using Microgenie DNA analysis software (Beckman). Hydrophilicity is represented with positive values. Amino acid number and one-letter symbols are plotted below the curve.

Approximately 2 μg of each primer was mixed with the reverse transcription product of 10 μg of Fao poly(A)+ mRNA, the PCR reaction was completed as described in Chapter 4 methods. Analyzing the PCR reaction by agarose/TBE gel electrophoresis, an approximately 600 bp DNA fragment was produced. This was excised from the gel, electroeluted, precipitated and blunt-ended, all as described in Chapter 4 methods. The blunt-ended insert was ligated into the blunt-ended Hinc II site of pUC19, the ligation was used to transform ampicillin-sensitive DH5-α competent cells, and four different white colonies were picked and used for plasmid preparations. The plasmid DNA from these 4 clones was purified using the mini-prep method described in Chapter 3 methods. The plasmids were then digested overnight with the restriction enzymes EcoR I and Hind III, the digested plasmids analyzed by gel electrophoresis, with the inserts, now EcoR I/Hind III-tailed, purified by electroelution. The tailed inserts were then ligated into the Hind III and EcoR I sites of the pGEM-9Z vector, the ligation products again used to transform ampicillin-sensitive DH5-α cells, with white colonies picked to produce purified pGEM plasmid containing the ASI cDNA. These plasmids were then transcribed *in vitro* as described, the RNA produced by these reactions were added to an *in vitro* rabbit reticulocyte translation mix in the presence of [^{35}S]-methionine and

-cysteine to radioactively label polypeptides synthesized by the translation reaction. A diagram illustrating the transcription and translation protocols is shown in Figure 4-13 along with the SDS-PAGE analysis of the in vitro translation products in Figure 4-14. Shown in Figure 4-14 is a pair of translation reactions using the "sense" and "antisense" RNA synthesized from the T7 and SP6 RNA polymerase promoter sites, respectively.

The ASI cDNA clone was used to perform a rat genomic DNA Southern blot analysis using restriction enzyme-digested rat DNA. Following our protocol presented in the Methods section, we probed rat DNA with the original 409 bp ASI cDNA insert isolated by the differential hybridization process. The enzymes EcoR I, Hind III, Pst I, and Xba I, all being enzymes that recognize and cut at a six bp sequence of DNA, were used to digest rat genomic DNA, and then the digested DNA was analyzed by the Southern procedure (Southern, 1975). The autoradiograph resulting from the blot indicated that with each enzyme, there were 10 to 15 DNA bands hybridizing to our 409 bp probe. A second Southern procedure performed on the same blot using a probe derived from the single copy gene, phosphoenolpyruvate carboxy-kinase (PEPCK), showed that the DNA was digested to completion, with one or two hybridizing bands displayed in each lane (data not shown). Subsequent blots (not shown)

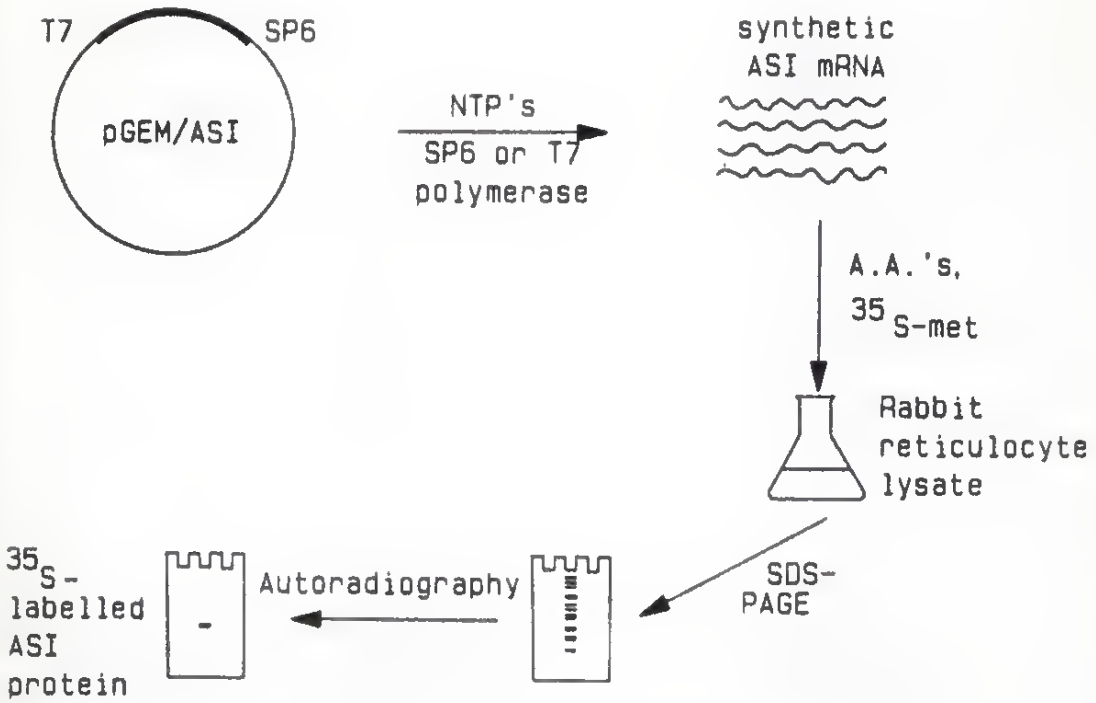

Fig 4-13. In vitro transcription and translation. Schematic diagram illustrates the procedure involved in making synthetic mRNA from a pGEM-based plasmid and the resultant in-vitro translation of the mRNA into a [S-35]-labelled protein.

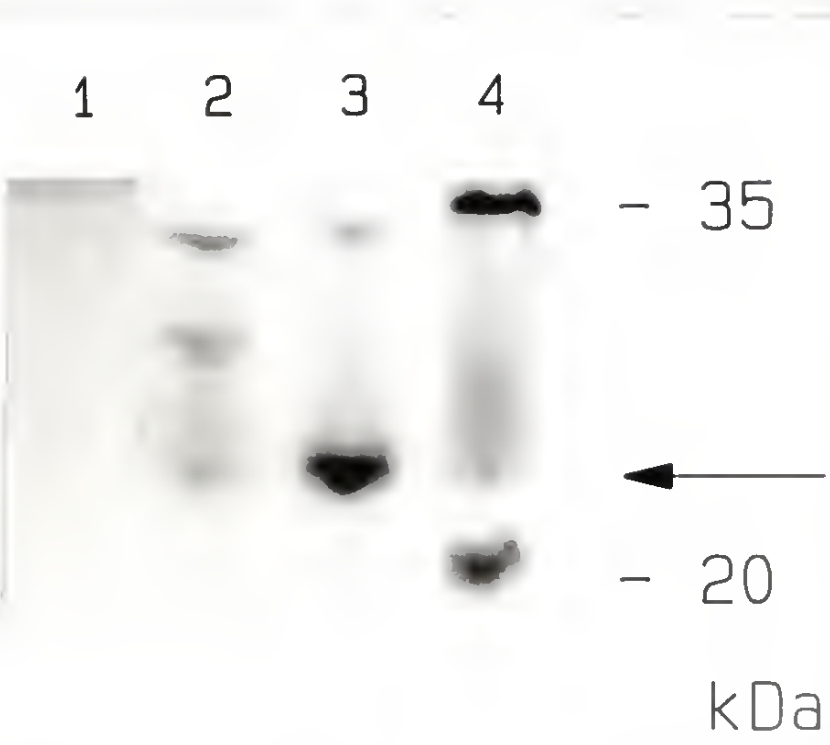

Figure 4-14. In vitro translation of rat Fao hepatoma ASI mRNA. SDS-PAGE analysis of in vitro translated mRNA synthesized from a pGEM-9Z plasmic containing the full-length ASI coding sequence. Lane 1, translation mix with no RNA added; lane 2, anti-sense RNA; lane 3, sense-RNA; lane 4, BMV RNA. The 2 BMV proteins shown in lane 4 are 35 and 20 kDa. Arrow marks location of putative ASI protein.

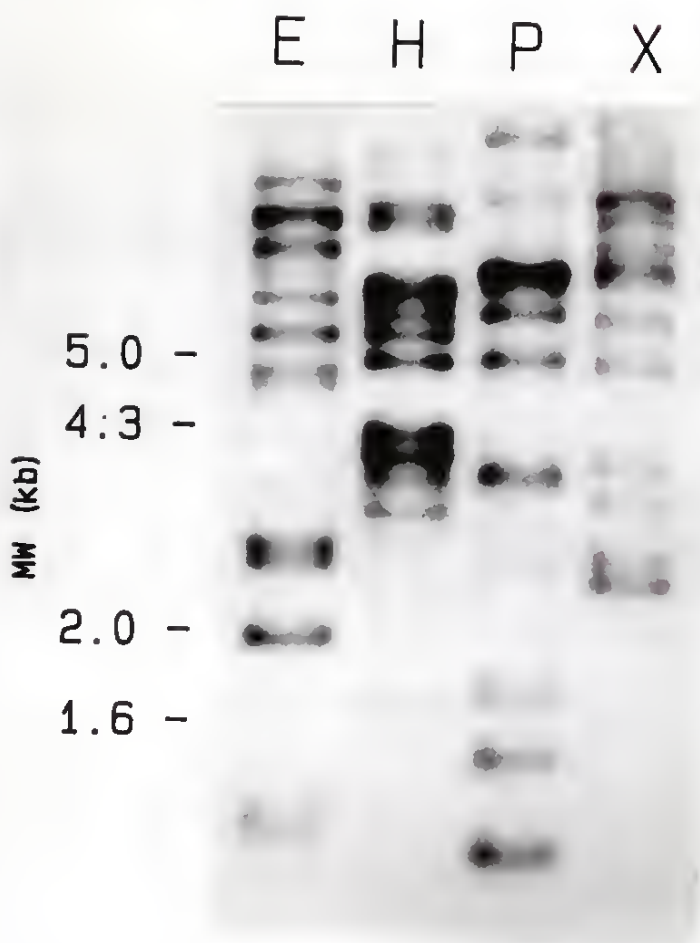

Figure 4-15. Rat genomic Southern analysis of the ASI gene. Rat genomic DNA was digested with EcoR I (E), Hind III (H), Pst I (P), and Xba I (X), subjected to electrophoresis, blotting and hybridization with radiolabelled ASI (S-5) cDNA. The resultant autoradiograph is shown above. Molecular size standards are shown at left. This same blot was probed with single copy gene PEPCK, demonstrating complete digestion of the rat DNA (data not shown).

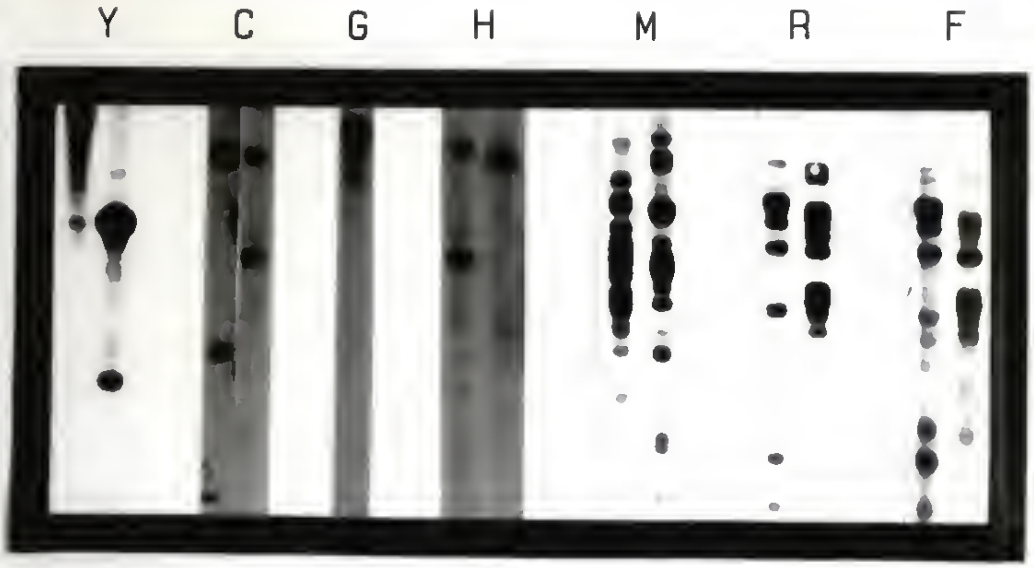

Fig. 4-16. Southern "Zoo" analysis of the ASI gene. Five micrograms of genomic DNA from various species or cells were digested with EcoR I or Pst I, size-fractionated by electrophoresis, blotted, and hybridized with radiolabelled ASI (S-5) cDNA probe. Resultant autoradiograph is shown above. Y, Yeast; C, Chicken; G, Alligator; H, Human; M, Mouse; R, Rat; F, Fao. Molecular size range of fragments is identical to those shown in Figure 4-15.

demonstrated that Southern procedures using a probe derived from an interior coding region of the ASI cDNA yielded one or a few bands per lane, but if the 3'-region of the cDNA was included, then the multiple band pattern would be observed.

Discussion

The characterizations of the ASI mRNA and corresponding cDNA were presented in this chapter. The ASI mRNA is relatively small at 612 bp, even with the addition of the approximately 100 residues of a typical poly(A) tail. The predicted coding region for the ASI polypeptide spans almost the entire length of the mRNA and would code for a polypeptide of over 21 kDa. It is possible that because there is very little non-coding region in both the 5'- and 3'-ends of the mRNA, that there may be few binding sites for regulatory proteins, as has been recently shown for the transferrin receptor and ferritin mRNAs (Theil, 1990). If this speculation is indeed true, then the induction of the ASI mRNA during amino acid starvation could be at least partially explained by proposing the absence of a regulatory site for enhanced degradation of mRNA during starvation on the ASI mRNA. If a majority of mRNAs did carry this turnover regulatory site, then the relative abundance of the ASI mRNA would increase due to increased turnover of other RNAs during the starvation situation. The ASI mRNA was

shown to be a cytosolic-associated mRNA, meaning that it is probably not a component of the plasma membrane amino acid transporter, System A which exhibits a similar form of amino acid-dependent regulation (Kilberg, (1986). However, as only integral membrane or secreted proteins are synthesized on membrane-bound polysomes, it is possible that this mRNA could encode a peripherally-associated membrane protein. Also, the cytosolic location of ASI mRNA synthesis does not mean the protein product remains in the cytosol, as it is known that mitochondrial, nuclear, and other subcellular organelles have their proteins translated from free polysomes, with the protein then being targeted to the correct destination (see e.g. Schatz and Butow, 1983).

Although neither the ASI DNA nor protein sequence are present in any of the data banks searched, the relatively high abundance of the ASI mRNA, as well as the fact that it was found in every rat tissue tested leads us to believe that the ASI gene product serves an essential function to the cell and may be ubiquitous. This, of course, is consistent with our initial observation that the mRNA remains intact during the crisis situation of total amino acid starvation when many other mRNAs nearly disappear.

It is difficult to draw any specific information from the relative abundances of the ASI mRNA seen in various rat tissues. As the highest abundance was seen in adipose

tissue, which has a specialized metabolism of its own centered about fatty acids, we may speculate that ASI is involved in fatty acid metabolism in some way. The low expression of ASI in liver was contrary to our results in the Fao hepatoma cell line, where the ASI mRNA was much more abundant. The significance of this difference is unclear.

Both the original cDNA fragment obtained from the differential hybridization of the hepatoma library as well as the 10 clones isolated from the normal rat liver library screening, using the hepatoma cDNA as a probe, resulted in isolation of incomplete length cDNAs. Although the primer extension reaction yielded primarily a full-length product, we did see some less abundant partial length extension products, perhaps caused by secondary structure in the 5'-region of the mRNA. After inspection of the 10 clones isolated from the rat liver library screening showed none of them to be full-length, we chose to obtain the full-length clone using the APCR method presented previously. We found the cloning method for primer extension products presented by Horlick et al., (1990) to be straightforward, and recommend that this method be used in parallel with primer extension to definitively analyze the 5'-end of mRNAs.

The ASI polypeptide is 21.4 kDa and as predicted, appears to be a largely hydrophilic protein with two regions of clustered positive charge. Potentially, these regions

may be important for maintaining secondary or tertiary structure, or could be involved in recognizing other molecules or as a targeting signal to a subcellular location. There are 4 cysteine residues in the predicted amino acid sequence, creating the potential for up to 2 intramolecular disulfide bonds. We do not know if there is any *in vivo* cleavage of a leader peptide off of the 5'-end of the ASI polypeptide. If not, the methionine residue at the 5'-end of the polypeptide would presumably result in a protein with a relatively long half-life as predicted by the N-end rule (Bachmair *et al.*, 1986). Cleavage of a leader peptide would create a new 5'-end residue which could change the *in vivo* half-life, depending on the amino acid exposed. The fact that the ASI polypeptide was easily detected by SDS-PAGE and autoradiographic analysis of *in vitro* translation in the rabbit reticulocyte lysate system suggests that the half-life of the ASI protein is on the order of hours and not minutes.

Perhaps the most intriguing results presented thus far have to do with the genomic analysis of the ASI gene. Our initial Southern blot of the ASI gene resulted in 10-15 bands hybridizing to the 409 bp original ASI insert. Our first interpretation of this result was that the DNA size-fractionated by electrophoresis was only partially digested, and the multiple banding pattern was an artifact. However,

the same blot was stripped, and rehybridized with a probe from the single-copy PEPCK gene, and this produced a high quality autoradiograph with one or two bands identified in each lane (data not shown). As the DNA was then judged to be completely digested, we proposed that the multiple banding pattern arose from one of two possible causes. First, the ASI gene may actually be a gene family, with a number of active or pseudogenes present in the genome. This pattern has been seen with the arginosuccinate synthetase gene, which is a single-copy gene in most species, but exists in multiple copies in human DNA. An alternative explanation is that the ASI gene is a single-copy gene, but somewhere within the 409 bp cDNA sequence that was used as a probe there is a small sequence that is a repetitive DNA element that occurs in the genome approximately 10-15 times (Singer, 1982). In this case, most of the bands identified by our Southern blot would not be related to the ASI gene except for the fact that they carried that small middle-repetitive element that was also present within the cDNA insert.

Additional genomic analyses were performed using DNA from various species. This "zoo" analysis or "zoo blot" illustrated that the multiple band hybridization pattern apparently is rodent specific. The multiple pattern was seen in the rat Fao cell line, in mouse, and in rat, but not

in human, chicken, alligator, or yeast genomic DNA. This observation could be explained by either a series of gene duplications within the rodent family, or if a repetitive element is involved, then that segment would be a rodent-specific sequence. A precedent for this latter explanation exists as demonstrated by the Alu family of repetitive elements, some of which are found only in the human genome (Schmid and Jelinek, 1982). On-going reasearch in our lab has indicated that the ASI gene is probably single or very low copy in rodent, and a short sequence near the 3'-end of the cDNA sequence is responsible for the multiple hybridization pattern.

The ASI mRNA and gene appear to be novel, as determined by the absence of any similar DNA or protein sequences in any known data bases. The determination of the function of the ASI gene product and its relationship to amino acid deprivation should prove to be an interesting quest.

CHAPTER 5

CONCLUSIONS AND FURTHER DIRECTIONS

After preparing and screening cDNA libraries from Fao rat hepatoma cells, one cDNA was identified that corresponded to an mRNA that is increased in concentration when the Fao cells were deprived of amino acids. This mRNA, called ASI, is relatively short, about 650 bp in length, and apparently encodes a 21 kDa polypeptide. The mRNA appears to be moderately abundant in the Fao cell line, being almost as abundant as actin as determined by Northern analysis. The mRNA is detectable in every tissue and cell line tested. The induction of this mRNA is between 2-to 3-fold over basal after a 9 to 12 hour starvation for total amino acids. Although the induction is blocked by the metabolic inhibitors cycloheximide and actinomycin D, it appears that the increase in ASI mRNA content is most likely due to reduced turnover of the ASI mRNA during starvation. This conclusion is based partly on the results of nuclear run-off transcription assays that show that the ASI gene is not transcriptionally activated to a significant degree during amino acid deprivation. There may be a separate protein acting as a stabilizing factor for the ASI mRNA, and when the transcription or translation of this factor is blocked by inhibitors of macromolecular synthesis during

amino acid starvation, the absence or reduction of this stabilizing protein presumably would not allow the ASI mRNA to accumulate as it does in the absense of the inhibitors. Although inhibitor studies may predict the existence of such mRNA-stabilizing proteins, only one such protein has been identified and purified. This is the 90 kDa mRNA-binding protein that acts as both an enhancer of transferrin translation as well as degradation of the transferrin receptor mRNA, apparently modulates both mRNA species by binding to the 5'- and 3'-ends of the mRNA, respectively (Theil, 1990). The discovery of this protein confirms predictions made up to 20 years ago based on inhibitor studies.

The ASI cDNA sequence has been entered into the GenBank DNA data base, and no significant homologies were found. Likewise, a search of known protein data bases using the predicted ASI polypeptide sequence have not yielded any similar protein sequences. As I believe ASI to be present in most cells and relatively abundant, I speculate that the ASI protein product serves a vital role in cellular function. The fact that the mRNA increases in content during amino acid starvation when many other mRNAs decrease in abundance during the same time period leads me to further speculate that the protein is not growth or cell cycle regulated, as we see mRNAs from this class (e.g. histone)

decrease in abundance during starvation. Since the mRNA increases during amino acid starvation, it is possible that ASI may be a non-integral membrane component or accessory to an amino acid transport system, some of which are known to be induced by amino acid deprivation (Kilberg, 1986). Alternatively, another likely role is that of a protease, which could serve to eliminate proteins unnecessary to a cell confronted with the amino acid-starved state and thereby release amino acids in the process. Another possibility would be that ASI serves some function related to the amino acid-tRNA synthetases. Increased activity of these complexes might be necessary during a time when the amino acid concentrations within the cell become lower. Perhaps ASI is serving some role in the metabolic conversions to or from amino acids in the cell, perhaps as an amino acid synthetase, or transaminase. Hopefully, future work on ASI will allow the identification of its role in cellular function.

One of the surprising outcomes of this work was the fact that only one cDNA clone was identified as an induced clone from a screening project using two separate cDNA libraries and inspection of over 60,000 independent cDNA clones. The fact that this single clone corresponded to an mRNA that had an increase of approximately 1.5-fold at the 3

hour time point brings the following observations and suggestions to mind:

1. The 1.5-fold increase in mRNA content suggests why this clone was not identified repeatedly, as might be expected for high-abundance clones -- a 1.5-fold increase is difficult to detect by the method of differential hybridization, and although the clone was likely to be appearing many times on the autoradiograms of the primary screenings, at the 1.5-fold level, it probably looked like an uninduced clone most of the time.

2. The expectation that amino acid starvation causes significant changes in the abundances of mRNAs in cells in culture at 3 hours into starvation may not be correct. Although the current model of adaptive regulation of System A-mediated transport suggests a starvation-related signal exists that promotes increased System A gene transcription (Kilberg, 1986; Moffett and Englesberg, 1984), we must remind ourselves that this model is based on results of inhibitor studies and genetic analyses and is not known with certainty. The results of protein and mRNA analyses introduced in Chapter 1 suggest few, if any, large scale changes in protein or mRNA composition are happening 3 to 6 hours after starvation begins.

Although the extracellular medium changes instantaneously when cells are transferred to culture in amino acid-free medium, the intracellular change in amino acid concentrations must decrease gradually from the values of the amino acid-fed state. The response to starvation cannot be expected to be instantaneous, as we might expect cells to respond to the application of a hormone that sets off a rapid metabolic cascade upon its application.

3. If the method of differential hybridization is to be considered as an approach to detect starvation-induced mRNAs, it would be advisable to choose a longer starvation period than 3 hours to isolate mRNA from fed and starved cells to prepare libraries and probes. Although one of the reasons the short 3 hour period was chosen was to eliminate screening for heat shock proteins, which are known to be induced by long periods of amino acid starvation, probes for known heat shock proteins are available, and any induced clones obtained from a screening could be cross screened against those probes to eliminate them from consideration of future study. Another recommendation would be to use subtracted probes in the differential screening, which should allow the detection of mRNAs less abundant by an order of magnitude than those detected by screening

with total plus and minus probes (Sargent, 1987). The use of biotinylated mRNAs and subtraction using phenol extraction has recently been introduced (Sive and St. John, 1988) and appears to produce a higher magnitude of subtraction than is achieved via hydroxyapatite chromatography. The utilization of this protocol could provide enough of an increase in sensitivity to make low abundance clones detectable by plus-minus screening. Certainly, the lack of an mRNA known to be induced by starvation left us without an important control often utilized by those performing differential hybridization in systems with previously identified induced mRNAs.

Along with the data presented in this work, we include here a brief discussion of work currently in progress or planned with ASI. Perhaps the approach that could provide the most new information regarding ASI would be the generation of antibodies to the ASI protein. With the full-length coding sequence of the protein already available, it should be a relatively straightforward process to express the protein in E. coli and purify adequate amounts of the protein to use as an antigen. Likewise, the production of synthetic peptides provides a relatively simple, although possibly costlier way to obtain purified polypeptide. This

method allows the specific synthesis of small regions of the protein to allow antibody production against separate epitopes of the protein. Generation of antibodies to the ASI protein would allow the following experiments to be done:

1. Identification of the ASI protein among the rest of cellular proteins using 1- or 2-dimensional SDS-PAGE followed by immunoblotting.
2. Induction of the ASI protein during starvation could be verified using immunoblotting of starved and fed cellular protein samples.
3. Tissue distribution of the ASI protein could be determined to compare to the mRNA distribution, again by immunoblotting.
4. Identification of the cellular location of the ASI protein could proceed either by immunoblotting of cell fractions representing purified cell fractions (e.g. nucleus, cytosol, etc.) or by immuno-electron microscopy. This work would also verify the cytosolic/membrane-bound determination.
5. Glycosylation of the ASI protein could be determined by treatment of cellular protein with and without a glycosidase, such as endoglycosidase H, followed by immunoblotting. Removal of an oligosaccharide group from the protein would give the

protein an slightly increased mobility that should be detectable after SDS-PAGE and immunoblotting.

We are currently collaborating with two groups in an attempt to identify the genomic location of the ASI gene. As the ASI gene appears to be single or low copy in human DNA, we have proceeded with in situ hybridization using [^{3}H]-ASI cDNA as a molecular probe to identify the chromosomal location of the ASI gene. This work is being conducted in the laboratory of Dr. Harry Ostrer in the Department of Pediatric Genetics, University of Florida College of Medicine.

For the rodent analysis, where a multiple (10-15) banding pattern is seen upon Southern analysis using genomic DNA digested with hexa-cutters, we have given the ASI probe to the laboratory of Dr. Edward Wakeland, Department of Pathology, University of Florida College of Medicine. Dr. Wakeland's laboratory is primarily interested in identifying the chromosomal locations corresponding to the multiple bands seen in the rodent genomic Southern blot for use as an aid to genomic mapping. As I believe the ASI gene to be low or single copy in rodent, many of the hybridizing bands are probably caused by the presence of a repetitive element within the ASI sequence. The location of the single or low copy gene in the rodent genome will be determined using a

smaller subfragment prepared from the ASI cDNA. The repetitive element must not be a part of this subfragment to be of use in locating the ASI gene.

REFERENCES

Ames, B. N. and Gary, B. (1959) Coordinate repression of the synthesis of four histidine biosynthetic enzymes by histidine. Proc. Natl. Acad. Sci. USA 45, 1453-1461.

Arndt, K. T., Styles, C. and Fink, G. R. (1987) Multiple global regulators control HIS4 transcription in yeast. Science 237, 874-880.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) Current protocols in molecular biology, John Wiley & Sons, Publishers, New York.

Aviv, H. and Leder, P. (1972) Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA 69, 1408-1412.

Bachmair, A., Finley, D., and Varshavsky, A. (1986) In vivo half-life of a protein is a function of its amino-terminal residue. Science 234, 179-186.

Barber, E. F., Handlogten, M. E. and Kilberg, M. S. (1983) Induction of amino acid transport System A is blocked by tunicamycin. J. Biol. Chem. 258, 11851-11855.

Beale, E. G., Hartley, J. L. and Granner, D.K. (1982) Dibutyryl cyclic AMP and glucose regulate the amount of messenger RNA coding for hepatic phosphoenolpyruvate carboxykinase (GTP). J. Biol. Chem. 257, 2022-2028.

Bernardi, G. (1965) Chromatography of nucleic acids on hydroxyapatite. Nature 206, 779-783.

Boerner, P. and Saier, M. H. (1985) Adaptive regulatory control of System A transport activity in a kidney epithelial cell line (MDCK) and a transformed variant (MDCK-T_1). J. Cell. Phys., 122, 308-315.

Bracy, D. S., Handlogten, M. E., Barber, E. F., Han, H.-P. and Kilberg, M. S. (1986) Cis-inhibition, trans-inhibition, and repression of hepatic amino acid transport mediated by System A. J. Biol. Chem. 261, 1514-1520.

Chiles, T. C., O'Brien T. W., and Kilberg, M. S. (1987) Production of monospecific antibodies to a low-abundance hepatic membrane protein using nitrocellulose immobilized protein as antigen. Anal. Biochem. 163, 136-142.

Chin, D. J., Gil, G., Faust, J. R., Goldstein, J. L., Browm, M. S. and Luskey, K. L. (1985) Sterols accelerate degradation of hamster 3-hydroxy-3-methylglutaryl coenzyme A reductase encoded by a constitutively expressed cDNA. Mol. Cell. Biol. 5, 634-641.

Church, G. and Gilbert, W. (1984) Genomic sequencing Proc. Natl. Acad. Sci. USA 81, 1991-1995.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156-159.

Cox, F., Gestautas, J., and Rapoport, B. (1988) Molecular cloning of cDNA corresponding to mRNA species whose steady state levels in the thyroid are enhanced by thyrotropin. J. Biol. Chem. 263, 7060-7067.

Davis, L. G., Dibner, M. D. and Battey, J. F. (1986) Basic methods in molecular biology, Elsevier Publishers, New York.

Decaux, J.-F., Antoine, B. and Kahn, A. (1989) Regulation of the expression of the L-type pyruvate kinase gene in adult rat hepatocytes in primary culture. J. Biol. Chem. 264, 11584-11590.

Dupret, J.-M., Brun, P., Perret, C., Lomri, N., Thomasset, M., and Cuisinier-Gleizes, P. (1987) Transcriptional and post-transcriptional regulation of vitamin D-dependent calcium-binding protein gene expression in the rat duodenum by 1,25-dihydroxycholecalciferol. J. Biol. Chem. 262, 16553-16557.

Durnam, D. and Palmiter, R. (1981) Transcriptional regulation of the mouse metallothionein-I gene by heavy metals. J. Biol. Chem. 256, 5712-5716.

Dworkin, M. B, and Dawid, I.B. (1980a) Construction of a cloned library of expressed embryonic gene sequences from Xenopus laevis. Dev. Biol. 76, 435-448.

Dworkin, M. B. and Dawid, I. B. (1980b) Use of a cloned library for the study of abundant poly (A)$^+$ RNA during Xenopus laevis development. Dev. Biol. 76, 449-464.

Ecker, J. R. and Davis, R. W. (1987) Plant defense genes are regulated by ethylene. Proc. Natl. Acad. Sci. USA 84, 5202-5206.

Englesberg, E. and Moffett, J. (1986) A Genetic approach to the study of neutral amino acid transport in mammalian cells in culture. J. Memb. Biol. 91, 199-212.

Feinberg, A. P. and Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132, 6-13.

Fafournoux, P., Remesy, C. and Demingne, C. (1983) Control of alanine metabolism in rat liver by transport processes or cellular metabolism. Biochem J. 210, 645-652.

Fong, A. D., Handlogten, M. E. and Kilberg, M. S. (1990) Substrate-dependent adaptive regulation and trans-inhibition of system A-mediated amino acid transport. Studies using rat hepatoma plasma membrane vesicles. Biochim. Biophys. Acta. 1022, 325-332.

Fornace, Jr., A. J., Alamo, Jr., I. and Hollander, M. C. (1988) DNA damage-inducible transcripts in mammalian cells. Proc. Natl. Acad. Sci. USA 85, 8800-8804.

Frohman, M. A., Dush, M. K., Martin, G. R. (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonuceotide primer. Proc. Natl. Acad. Sci. USA 85, 8998-9002.

Garnier, J., Osguthorpe, D. J. and Robson, B. (1978) Analysis of the accuracy and implications for predicting the secondary structure of globular proteins. J. Mol. Biol. 120, 97-120.

Gazzola, G. C., Dall'Asta, V., Franchi-Gazzola, R. and White, M. F. (1981) The cluster-tray method for rapid measurement of solute fluxes in adherent cultured cells. Anal. Biochem. 115, 368-373.

Gazzola, G.C., Franchi, R., Saibene, V., Ronchi, P. and Guidotti, G. G. (1972) Regulation of amino acid transport in chick embryo heart cells. Biochim. Biophys. Acta 266, 407-421.

Goldberg, I. H. and Friedman, P. A. (1971) Antibiotics and nucleic acids. Ann. Rev. Bch. 40, 775-810.

Goodridge, A. G. (1987) Dietary regulation of gene expression: enzymes involved in carbohydrate and lipid metabolism. Ann. Rev. Nutr. (Olson, R. E., Beutler, E. and Broquist, H. P. eds.) 7, 157-185.

Gribskov, R. (1984) The codon prefernce plot: graphic analysis of protein coding sequences and prediction of gene expression. Nucl. Acids Res. 12, 539-549.

Groudine, M, Peretz, M., and Weintraub, H. (1981) Transcriptional regulation of hemoglobin switching on chicken embryos. Mol. Cell. Biol. 19, 317-331.

Grunstein, M. and Hogness, D. S. (1975) Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA 72, 3961-3965.

Gubler, U. and Hoffman, B. (1983) A simple and very effective method for generating cDNA libraries. Gene 25, 263-269.

Han, J. H., Stratowa, C. and Rutter, W. J. (1987) Isolation of full-length putative rat lysophospholipase cDNA using improved methods for mRNA isolation and cDNA cloning. Biochemistry 26, 1617-1625.

Hanahan, D. (1983). Studies on the transformation of E. coli with plasmids. J. Mol. Biol. 166, 557-580.

Handlogten, M. E., Weissbach, L. and Kilberg, M. S. (1982) Heterogeneity of Na^+-independent 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid and L-leucine transport in isolated rat hepatocytes in primary culture. Biochem. Biophys. Res. Comm. 104, 307-313.

Hinnebusch, A. G. (1986) The general control of amino acid biosynthetic genes in the yeast Saccharomyces cerevisiae. CRC Crit. Rev. Biochem. 21, 277-317.

Hinnebusch, A. G. (1988) Mechanisms of gene regulation in the general control of amino acid biosynthesis in Saccharomyces cerevisiae. Microbiol. Rev. 52, 248-273.

Hope I. A. and Struhl, K. (1986) Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. Cell, 46, 885-894.

Horlick, K. R., Cheng, I. C., Wong, W. T., Wakeland, E. K. and Nick, H. S. (1990) Genomic structure and chromosomal assignment of the mouse lipocortin I gene. Genomics (submitted for review).

Huynh, T. V., Young, R. A. and Davis, R. W. (1985) Construction and screening cDNA libraries. In DNA cloning, a practical approach, Vol. I. D. M. Glover, Ed. IRL Press, Oxford/Washington D.C.

Ish-Horowicz, D. and Burke, J. (1981) Rapid and efficient cosmid cloning. Nucl. Acids Res. 9, 2989-2998.

Karin, M., Haslinger, A., Holtgreve, H., Richards, R. I., Krauter, P., Westphal, H. M. and Beato, M. (1984) Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-IIA gene. Nature 308, 513-519.

Kelley, D. S. and Potter, V. R. (1978) Regulation of amino acid transport by amino acid depletion and supplementation in monolayer cultures of rat hepatocytes. J. Biol. Chem. 253, 9009-9017.

Kilberg, M. S. (1986) System-A mediated amino acid transport: metabolic control at the plasma membrane. Trends in Biochem. Sci. 11, 183-186.

Kilberg, M. S., Han, H.-P., Barber, E. F. and Chiles, T. C. (1985) Adaptive regulation of neutral amino acid transport System A in rat H4 hepatoma cells. J. Cell. Physiol. 122, 290-298.

Kilberg, M. S., Vida, T. A. and Barber, E. F. (1983) Regulation of neutral amino acid transport in hepatocytes isolated from adrenalectomized rats. J. Cellular Physiol. 114, 45-52.

King, P. V. and Blakesley, R. W. (1986) Effect of polyethylene glycol on DNA ligations. Focus (Bethesda, Md.) 8, 1.

Kraft R., Tardiff, J., Krauter, K. S. and Leinwand, L. A. (1988) Using mini-prep plasmid DNA for sequencing double stranded templates with sequenase. Biotechniques 6, 544-547.

Kyte T. and Doolittle J. (1982) A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157, 105-132.

Lazard, M., Mirande, M. and Walker, J.-P. (1987) Expression of the aminoacyl-tRNA synthetase complexes in cultured Chinese hamster ovary cells. J. Biol. Chem. 262, 3982-3987.

Levinson, W., Kravitz, S. and Jackson, J. (1980) Amino acid deprivation induces synthesis of four proteins in chick embryo cells. Exp. Cell. Res. 130, 459-463.

Lin, L.-W. and Lee, F.-J. (1984) Induction of two genes by glucose starvation in hamster fibroblasts. Proc. Natl. Acad. Sci. USA 81, 988-992.

Loh E. Y., Elliot, J. F., Cwirla, S, Lanier, L. L., and Davis, M. M. (1989) Polymerase chain reaction with single-sided specificty: analysis of T cell receptor δ chain. Science 243, 217-243.

Loros, J. J., Denome., S. A. and Dunlap, J. C. (1989) Molecular cloning of genes under control of the circadian clock in Neurospora. Nature 348, 385-388.

Lucchini, G., Hinnebusch, A. G., Chen, C. and Fink, G. R. (1984) Positive regulatory interactions of the HIS4 gene of Saccharomyces cerevisiae. Mol. Cell. Biol. 4, 1326-1333.

Magnuson, M. A., Dozin, B. and Nikodem, V. M. (1985) Regulation of specific rat liver messenger ribonucleic acids by triiodothyronine. J. Biol. Chem. 260, 5906-5912.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) Molecular cloning: A laboratory manual. Cold Spring Harbor Publishers, New York.

Marzluff W. F. and Huang R. C. C. (1985) Transcription and translation: A practical approach. (B. D. Hames and S. J. Higgins, Eds.) pp. 89-129. IRL Press, Oxford.

Masiakowski, P., Breathnach, R., Bloch, J., Gannon, F., Krust, A. and Chambon, P. (1982) Cloning of cDNA sequences of hormone-regulated genes from the MCF-7 human breast cancer cell line. Nucleic Acids Res. 10, 7895-7903.

Maxam, A. and Gilbert, W. (1977) A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA 74, 560-564.

McCormick, C. C., Menard, M. P. and Cousins, R. J. (1981) Induction of hepatic metallothionein by feeding zinc to rats of depleted zinc status. Am. J. Physiol 248, E414-E421.

McKnight, S. L. and Kingsbury, R. (1982) Transcription control signals of a eukaryotic protein-coding gene. Science 217, 316-324.

Mechler, B. M. (1987) Isolation of messenger RNA from membrane-bound polysomes. Meth. Enzymol. 152, 241-248.

Merrington, M. (1941) Table of percentage points of the t-Distribution. Biometrika 32, 300.

Messing J. (1983) New M13 vectors for cloning. Meth. Enzymol. 101, 20-78.

Messing J., Gronenborn, B., Muller-Hill, B. and Hofschneider, P. H. (1977) Filamentous coliphage M13 as a cloning vehicle: insertion of a Hind III fragment of the lac regulatory region in M13 replicative form in vitro. Proc. Natl. Acad. Sci. USA 74, 3642-3646.

Messenguy, F. and Delforge, J. (1976) Role of transfer ribonucleic acids in the regulation of several biosyntheses in Saccharomyces cerevisiae. Eur. J. Biochem. 67, 335-339.

Miller, J. H and Reznikoff, W. S. (1978) The operon Cold Spring Harbor Publishers, New York.

Misrahi, M., Atger, M. and Milgrom, E. (1987) A novel progesterone-induced messenger RNA in rabbit and human endometria. Cloning and sequence analysis of the complementary DNA. Biochem. 26, 3975-3982.

Moffett, J. and Englesberg, E. (1984) Recessive constitutive mutant Chinese hamster ovary cells (CHO-K1) with an altered A system for amino acid transport and the mechanism of gene regulation of the A system. Mol. Cell. Biol. 4, 799-808.

Morley, J. E., Mooradian, A. D., Silver, A. J., Heber, D. and Alfin-Slater, R. B. (1988) Nutrition in the elderly. Ann. Intern. Med. 1988, 890-904.

Mueller, P. P., Harashima, S. and Hinnebusch, A. G. (1987) A segment of GCN4 mRNA containing the upstream AUG codons confers translational control upon a heterologous transcript. Proc. Natl. Acad. Sci. USA 84, 2863-2867.

Mueller, P. P. and Hinnebusch, A. G. (1986) Multiple upstream AUG codons mediate translational control of GCN4. Cell 45, 201-207.

Munro S. and Pelham, H. R. B. (1986) An hsp-like protein in the endoplasmic reticulum. Cell, 46, 291-300.

Niederberger, P., Aebi, M. and Huetter, R. (1983) Influence of the general control of amino acid biosynthesis on cell growth and cell viability in Saccharomyces cerevisiae. J. Gen. Microbiol. 129, 2571-2583.

Osborn, T. F., Gil, G., Brown, M. S., Kowal, R. C. and Goldstein, J. L. (1987) Identification of promoter elements required for in-vitro transcription of hamster 3-hydroxy-3-methylglutaryl coenzyme A reductase gene. Proc. Natl. Acad. Sci. USA 84, 3614-3618.

Osborn, T. F., Goldstein, J. L. and Brown, M. S. (1985) 5' End of HMG CoA reductase contains sequences responsible for cholesterol-mediated inhibition of transcription. Cell 42, 203-212.

Oxender, D. L. and Christensen, H. N. (1963) Distinct mediating systems for the transport of neutral amino acids by the Ehrlich cell. J. Biol. Chem. 238, 3686-3699.

Riggs, T. R. and Pan M. W. (1972) Transport of amino acids in newborn rat renal cortex cell. Biochem. J. 128, 19-27.

Pardee, A. B., Jacob, F. and Monod, J. (1959) The genetic control and cytoplasmic expression of inducibility in the synthesis of ß-galactosidase of E. coli. J. Mol. Biol. 1, 165-178.

Pestka, S. (1971) Inhibitors of ribosome functions. Ann. Rev. Bch. 40, 697-710.

Poso, A. R., Wer, J. J. and Mortimore, G. E. (1982) Multi-functional control by amino acids of deprivation-induced proteolysis in liver: Role of leucine. J. Biol. Chem. 257, 12115-12120.

Rowekamp, W. and Firtel, R. A. (1980) Isolation of developmentally regulated genes from Dictyostelium. Dev. Biol. 79, 409-418.

Saito, H., Kranz, D. M., Takagaki, Y., Hayday, A. C., Eisen, H. N. amd Tonegawa, S. (1984) Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences. Nature 309, 757-762.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-5467.

Sargent, T. D. (1987) Isolation of differentrially expressed genes. Meth. Enzymol. 152, 423-432.

Schatz, G. and Butow, R. A. (1983) How are proteins imported into mitochondria? Cell 32, 316-318.

Schmid, C. W. and Jelinek, W. R. (1982) The Alu family of dispersed repetitive sequences. Science 216, 1065-1070.

Schneider, C., King, R. M., and Philipson, L. (1988) Genes expressed at growth arrest of mammalian cells. Cell 54, 787-793.

Singer, M. F. (1982) SINES and LINES: highly repeated short and long interspersed sequences in mammalian genomes. Cell 28, 433-434.

Sive, H. L. and St. John, T. (1988) A simple subtractive technique employing photoactivatable biotin and phenol extraction. Nucl. Acids Res. 16, 10937.

Smith G. E. and Summers M. D. (1980) The bidirectional transfer of DNA and RNA to nitrocellulose or diazo-benzyloxymethyl-paper. Anal. Biochem 109, 123-129.

Sobczak, J., Lotti, A.-M., Taroux, P. and Duguet, M. (1987) Molecular cloning of mRNA sequences transiently induced during rat liver regeneration. Exper. Cell Res. 169, 47-56.

Solomon, D. H., Raynal, M.-C., Tejwani, G. A. and Cayre, Y. E. (1988) Activation of the fructose 1,6-bisphosphatase gene by 1,25-dihydroxyvitamin D_3 during monocytic differentiation. Proc. Natl. Acad. Sci. USA 85, 6904-6908.

Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517.

Staden P. (1984) Measurements of the effects that coding for a protein has on DNA sequence and their use for finding genes. Nucl. Acids Res. 12, 551-567.

St. John, T. P. and Davis, R. W. (1979) Isolation of galactose-inducible DNA sequences from Saccharomyces cerevisiae by differential plaque filter hybridization. Cell, 16, 443-452.

Struhl, K. (1982) Regulatory sites for his3 expression in yeast. Nature 300, 284-286.

Swerdel, M. R. and Cousins, R. J. (1982) Induction of kidney metallothionein and metallothionein messenger RNA by zinc and cadmium. J. Nutr. 112, 801-809.

Theil, E. C. (1990) Regulation of ferritin and transferrin receptor mRNAs. J. Biol. Chem. 265, 4771-4774.

Thireos, G., Driscoll Penn, M. and Greer, H. (1984) 5' Untranslated sequences are required for the translational control of a yeast regulatory gene. Proc. Natl. Acad. Sci. USA 81, 5096-5100.

Thomas, P. S. (1980) Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA 77, 5201-5205.

Timberlake, W. E. (1980) Developmental gene regulation in Aspegillus nidulans. Dev. Biol. 78, 497-510.

Walker, P. S., Donovan, J. A., VanNess, B. G., Fellows, R. E. and Pessin, J. E. (1988) Glucose-dependent regulation of glucose transport activity, protein, and mRNA in primary cultures of rat brain glial cells. J. Biol. Chem. 263, 15594-15601.

Wallner, B. P., Mattaliano, R. J., Hession, C., Cate, R. L., Tizard, R., Sinclair, L. K., Foeller, C., Chow, E. P., Browning, J. L., Ramachandran, K. L., and Pepinsky, R. B. (1986) Cloning and expression of human lipocortin, a phospholipase A_2 inhibitor with potential anti-inflammatory activity. Nature, 320, 77-81.

Williams, J. G. and Lloyd, M. M. (1979) Changes in the abundance of polyadenylated RNA during slime mold development using cloned molecular hybridization probes. J. Mol. Biol. 129, 19-35.

Young, R. and Davis, R. (1983) Efficient isolation of genes using antibody probes. Proc. Natl. Acad. Sci. USA 80, 1194-1198.

Zalkin, H. and Yanofsky, C. (1982) Yeast gene TRP5: structure, fuction, and regulation. J. Biol. Chem. 257, 1491-1500.

Zurita, M., Bieber, D., Ringold, G. and Mansour, T. E. (1987) Cloning and characterization of a female genital complex cDNA from the Liver fluke Fasciola hepatica. Proc. Natl. Acad. Sci. USA 84, 2340-2344.

BIOGRAPHICAL SKETCH

Neil Frank Shay was born on October 16, 1954, in Fairfield, Connecticut. After graduating from Roger Ludlowe High School in 1972, he entered the University of Massachusetts at Amherst, and received a Bachelor of Science degree in zoology, graduating magna cum laude in 1976. In 1977, he entered the physics program at the University of Massachusetts, receiving a Master of Arts degree in 1979. September 1979 began a six-year period during which Neil was a public high school physics and biology teacher, as well as a coach at Kennett High School, Conway, New Hamphire and Yarmouth High School, Yarmouth, Maine. In August 1985, he enrolled at the University of Florida to pursue a doctoral degree under the direction of Dr. Michael S. Kilberg of the Department of Biochemistry and Molecular Biology. After graduating in August, 1990, he plans to join the laboratory of Dr. Robert Cousins as a post-doctoral fellow. Neil was married in 1981 to Elizabeth Susan Moore. Neil and Elizabeth have a daughter, Laura Jane, born January, 1988.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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